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From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

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in its capacity as elected Office

Date of mailing (day/month/year) 14 January 1999 (14.01.99)	
International application No. PCT/GB98/01382	**Applicant's or agent's file reference** DUNW/19095PC
International filing date (day/month/year) 27 May 1998 (27.05.98)	**Priority date** (day/month/year) 27 May 1997 (27.05.97)
Applicant KAY, Richard, Andrew	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

21 December 1998 (21.12.98)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

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REC'D 05 AUG 1999

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
DUNW/19095PC		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/GB98/01382	27/05/1998	27/05/1997
International Patent Classification (IPC) or national classification and IPC		
C07K14/725		
Applicant		
UNIVERSITY OF DUNDEE et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 9 sheets, including this cover sheet.

   ☐ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which hav been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☒ Certain documents cited
   - VII ☒ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
21/12/1998	02.08.99
Name and mailing address of the international preliminary examining authority:	Authorized officer
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Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/GB98/01382

I. Basis of th r port

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-53 as originally filed

Claims, No.:

1-20 as originally filed

Drawings, sheets:

1/16-13/16, 15/16-16/16 as originally filed

2. The amendments have resulted in the cancellation of:

☐ the description, pages:
☐ the claims, Nos.:
☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

☐ the entire international application.

☒ claims Nos. 18-20.

because:

☒ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):

see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☐ no international search report has been established for the said claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-17
	No:	Claims	18-20
Inventive step (IS)	Yes:	Claims	1-17
	No:	Claims	18-20
Industrial applicability (IA)	Yes:	Claims	1-17
	No:	Claims	

2. Citations and explanations

see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

VII. Certain defects in the international application

The following defects in the form or contents of the international application have been noted:

see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Part I

Pages filed on 20.10.1998: Under Rule 13ter.1(f) PCT, sequence listings filed after the filing date of the application, which are not filed as amendments, do not form part of the description and will not be annexed to this report.

Part III

Claims 18-20 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Art. 34(4)(a)(i) PCT).

Part V

Reference is made to the following documents:

D1 WO-A-94 14067
D2 Journal of Experimental Medicine, 179(2), 1994, 413-424
D3 WO-A-93 04695
D4 WO-A-92 12260
D5 WO-A-98 01738

a) Prior art

D1 discloses a method of identifying the V gene usage of T cells which are responsive in sarcoidosis patients. The method comprises the following steps (see p. 47-62):

- bronchoalveolar samples from sarcoidosis patients (i.e. samples containing T cells which have responded to the antigen) and samples from healthy subjects are obtained
- The T cells are incubated with TCR specific monoclonal antibodies (with specificity for the Vα or Vβ region) and the percentage of sick and healthy subjects having a specific TCR is compared.

Furthermore, this document shows a method of treating sarcoidosis by administering antibodies with specificity for the sarcoidosis specific TCR (anti-TCR antibody treatment, see chapter 5.3) or by administering a peptide comprising an amino acid sequence of the specific TCR (peptide immunization, see chapter 5.4).

D2 discloses an analysis of Vβ usage by CD4+ and CD8+ T cells from HIV infected individuals in response to an in vitro stimulation with the superantigen ETA of streptococcus pyrogenes (see summary). The method comprises the following steps:

- peripheral blood is obtained and stimulated with the bacterial superantigen
- the T cells are stained with monoclonal antibodies specific for human TCR V region epitopes. A comparison of T cell subpopulations bearing a given Vβ after in vitro antigen stimulation is calculated as percentage of T cell blasts bearing a particular Vβ after stimulation divided by the percentage of T cells bearing the Vβ before stimulation (see Material and Methods).

D3 discloses a method of treating rheumatoid arthritis in a mammal by (see claim 1):

- obtaining a sample of synovium
- identifying TCR variable regions
- administering an effective amount of antibodies to one of said TCR variable regions.

This document shows also a method of immunizing to prevent rheumatoid arthritis by administering selected TCR variable regions (see claim 7).

D4 discloses a method for describing repertoires of TCR by the following steps:

- Reverse transcription of RNA in a sample or extraction of sample DNA
- obtaining by PCR an elongation product corresponding to the (V,C)J region
- analysing the size of the elongation product.

D4 is not concerned with quantification of specific TCR per T cells.

b) Novelty and Inventive step

The subject-matter of claim 1 is distinguished from the most relevant prior art, which is represented by either of D1 or D2, by the following feature:

The method of claim 1 not only quantifies the number of T cells in a subset having a specific TCR before and after antigen stimulation, but measures the increase of TCR **expression per specific T cell subset**. Claim 1, therefore meets the requirements of Art. 33(2) PCT. By the distinguishing feature, one can identify T cells which have been specifically stimulated by an antigen, since antigen interaction results in internalization of the TCR and concomitant expression of new TCR, i.e. increase in TCR mRNA levels. The measurement is not disturbed by unspecific cytokine-mediated activation, as this does not lead to TCR internalization and concomitant increased TCR expression. The results of the prior art methods, however, may become disturbed by cytokine-triggered antigen-independent T cell activation.

None of the prior art documents would give the skilled person an incentive to modify the method of D1 and D2 in order to achieve more reliable results. Therefore, independent claim 1 appears to be inventive (Art. 33(3) PCT).

Dependent claims 2-17 contain preferred embodiments of the novel and inventive idea embodied in claim 1 and would also appear to meet the requirements of Articles 33(2) and (3) PCT.

Claims 18-20 are not novel for the following reasons (Art. 33(2) PCT):
Claim 18 relates to a method of treating a patient. It is characterized by the method step of administering an agent, step (b) in claim 18. This step is already known from D1 and D3.
The step (a) characterizes the identification of the disease-causing T cell subset and not the method of eliminating this specific subset. The method of identifying the cause of a disease cannot establish the novelty of a method of treating the disease.
Therefore, since the method of administering an agent to ameliorate a disease is not novel, the subject-matter of claim 18 is not novel.
Claims 19 and 20 define the treatment step more precisely. However, the features are also known from D1 and D3. Thus claims 19 and 20 are not novel either.

c) Industrial Applicability

For the assessment of the present claims 18-20 on the question whether they are industrially applicable, no unified criteria exist in the PCT. The patentability can also be

dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Part VI

D5, published on 15.01.1998, discloses a method of diagnosing a disease associated with microbial infections and comprises the following steps:

- obtaining a sample comprising T cells from a subject suspected of being afflicted by the disease
- reverse transcription of **TCR mRNA**
- determining the most abundant TCR fragment
- comparison with a library which comprises abundant TCR fragments in connection with the disease diagnosis for determining any similarities
- from determination of similarities diagnosing the disease of he patient.

This document therefore, could make obvious the subject-matter of claims 1-20. However, it is assumed that the priority date of the present application is validly claimed. The present priority date of 27.05.1997 is, namely, before the publication date of D5.

Part VII

a) Contrary to the requirements of Rule 5.1(a)(ii) PCT, the relevant background art disclosed in the documents D1 and D3 is not mentioned in the description, nor are these documents identified therein.

b) The expression "herein incorporated by reference" or equivalents thereof (see e.g. pages 18, 25 and 26) will have to be deleted in some cases when entering the national or regional phase (see e.g. the Guidelines for Examination in the EPO, C-II, 4.18)

Part VIII

The last paragraph on p. 46 is hard to understand as Fig. 13 (14/16) which should go with it, is missing (Art. 6 PCT).

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Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ANALOGUES OF hGH-RH (1-29)NH_2 HAVING ANTAGONISTIC ACTIVITY

(57) Abstract

Synthetic analogues of hGH-RH(1-29)NH_2 having substitutions of various amino acids and acylated at the N-terminus, and exhibiting prolonged antagonistic duration. Embodiments include analogues of the formula: X-R^1-R^2-R^3-R^4-R^5-R^6-Thr-R^8-Ser-Tyr-R^{11}-R^{12}-Val-Leu-R^{15}-Gln-Leu-Ser-R^{19}-R^{20}-R^{21}-Leu-Leu-Gln-Asp-Ile-R^{27}-R^{28}-R^{29}, wherein X is nil, H, Ac, IAc, BrProp, For, Ibu, Nac, 2-Nac, 1- or 2-Npt, 1- or 2-Npr or Aqc; R^1 is Tyr, His, Glu or Glt; R^2 is D-Arg, D-Cit, D-Har, D-Lys or D-Orn; R^3 is Asp, Ala or Gly; R^4 is Ala or Gly; R^5 is Ile, Ala or Gly; R^6 is Phe, Ala, Pro, Tpi, Nal or Phe(Y), in which Y is F, Cl, Br, NO_2, CH_3 or OCH_3; R^8 is Asn, Ser, Val, Ile, Ala, Abu, Nle or Aib; R^{11} is Arg, D-Arg or Cit; R^{12} is Lys, D-Lys Cit or Ala; R^{15} is Gly, Ala, Abu or Gln; R^{19} is Ala or Abu; R^{20} is Arg, D-Arg or Cit; R^{21} is Lys, D-Lys or Cit; R^{27} is Met, Nle or Abu; R^{28} is Ser, Asn, Asp or Abu; R^{29} is Agm, Arg-NH_2, Arg-OH, Cit-NH_2, Cit-OH, Har-NH_2 or Har-OH; provided that when R^1 is Glt, X is nil and when X is H, R^{15} is other than Gly, and pharmaceutically acceptable acid addition salts thereof.

see back of

In several of these investigations, it was found that variants of the hGH-RH agonistic analogues had antagonistic, rather than agonistic, activity. Thus, in US 4,659,693 (where R^2 may be certain D-Arg residues substituted with alkyl groups), when R^1 is H, the hGH-RH analogues are said to act as antagonists. Similarly, in WO 91/16923, discussed above, if R^2 in the analogues is D-Arg, and R^8, R^9, and R^{15} are substituted as indicated above, antagonistic activity is said to result. These antagonistic peptides are said to be suitable for administration as pharmaceutical compositions to treat conditions associated with excessive levels of GH, e.g., acromegaly.

The antagonistic activity of the hGH-RH analogue "[Ser^9-Ψ[CH_2-NH]-Tyr^{10}]hGH-RH(1-29)" of US Patent 5,084,555 was said to result from the pseudopeptide bond (i.e., a peptide bond reduced to a [CH_2-NH] linkage) between the R^9 and R^{10} residues. (It is noted that although this patent employed the seemingly redundant "Ψ[CH_2-NH]" formula for the pseudopeptide bond, actually only one such linkage had been introduced into the peptide.) However, the antagonistic properties of [Ser^9-Ψ[CH2-NH]-Tyr^{10}] hGH-RH(1-29) were said to be inferior to a conventional antagonist, [N-Ac-Tyr^1, D-Arg^2]GH-RH(1-29)-NH_2.

SUMMARY OF THE INVENTION

There is provided a novel series of synthetic analogues of hGH-RH(1-29)NH_2. These analogues inhibit the activity of endogenous hGH-RH, and therefore prevent the release of growth hormone. This inhibition is believed to result from replacement of various amino acids and acylation with aromatic or nonpolar acids at the N-terminus of GH-RH(1-29)NH_2. The analogues exhibit prolonged antagonistic duration.

Specifically, the invention relates to peptides comprising the formula:

X-R^1-R^2-R^3-R^4-R^5-R^6-Thr-R^8-Ser-Tyr-R^{11}-R^{12}-Val-Leu-R^{15}-Gln-Leu-Ser-R^{19}-R^{20}-R^{21}-Leu-Leu-Gln-Asp-Ile-R^{27}-R^{28}-R^{29}

wherein

X is nil, H, Ac, IAc, BrProp, For, Ibu, Nac, 2-Nac, 1- or 2-Npt, 1- or 2-Npr or Aqc,

R^1 is Tyr, His, Glt or Glu,

R^2 is D-Arg, D-Cit, D-Har, D-Lys or D-Orn,

R^3 is Asp, Ala or Gly,

R^4 is Ala or Gly,

R^5 is Ile, Ala or Gly,

R^6 is Phe, Ala, Pro, Tpi, Nal, or Phe(Y), in which Y is F, Cl, Br, NO_2, CH_3 or OCH_3,

R^8 is Asn, Ser, Val, Ile, Ala, Abu, Nle, or Aib,

R^{11} is Arg, D-Arg or Cit,

R^{12} is Lys, D-Lys, Cit or Ala,

R^{15} is Gly, Ala, Abu or Gln,

R^{19} is Ala or Abu,

R^{20} is Arg, D-Arg or Cit,

R^{21} is Lys, D-Lys or Cit,

R^{27} is Met, Nle or Abu,

R^{28} is Ser, Asn, Asp or Abu,

R^{29} is Agm, Arg-NH_2, Arg-OH, Cit-NH_2, Cit-OH, Har-NH_2 or Har-OH,

provided that when R^1 is Glt, X is nil, and when X is H, R^{15} is other than Gly,

and pharmaceutically acceptable acid addition salts thereof.

Among the preferred embodiments are peptides wherein X is H and R^{15} is Abu; or wherein X is Nac, For, or Ibu, R^1 is Tyr or His, R^2 is D-Arg or D-Cit, R^3 is Asp, R^4 is Ala, R^5 is Ile, R^6 is Phe(pCl) or Nal, R^{11} is Arg, R^{12} is Lys, R^{15} is Abu or Ala, R^{19} is Ala or Abu, R^{20} is Arg, R^{21} is Lys, R^{27} is Nle, R^{28} is Ser or Asp, and R^{29} is Agm or Arg-NH_2. Three very preferred embodiments hav th formulae:

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ANALOGUES OF hGH-RH(1-29)NH_2 HAVING ANTAGONISTIC ACTIVITY

FIELD OF THE INVENTION

This invention was made in part with Government support from the Medical Research Service of the Veterans Affairs Department. The Government has certain rights in this application.

The present invention relates to novel synthetic peptides which inhibit the release of growth hormone from the pituitary in mammals, and to therapeutic compositions containing these novel peptides.

BACKGROUND OF THE INVENTION

Growth Hormone ("GH") is a peptide having 191 amino acids which stimulates the production of numerous different growth factors IGF-I and so promotes growth of numerous tissues (skeleton, connective tissue, muscle and viscera) and physiological activities (raising nucleic acid and protein synthesis and lipolysis, but lowering urea secretion).

Release of GH is under the control of releasing and inhibiting factors secreted by the hypothalamus. The primary releasing factor is growth hormone releasing hormone ("GH-RH"); human growth hormone-releasing hormone ("hGH-RH") is a peptide having 44 amino acids. The novel peptides of the present invention relate to analogues of hGH-RH having only residues 1 through 29 ("hGH-RH(1-29)NH_2"), i.e., to analogues of the peptide which has the amino acid sequence:

Tyr-Ala-Asp-Ala-Ile^5-Phe-Thr-Asn-Ser-Tyr^{10}-Arg-Lys-Val-Leu-Gly^{15}-Gln-Leu-Ser-Ala-Arg^{20}-Lys-Leu-Leu-Gln-Asp^{25}-Ile-Met-Ser-Arg^{29}-NH_2

GH has been implicated in several diseases. One disease in which GH is involved is acromegaly, in which excessive levels f GH are present. Th abnormally enlarged facial and extr mity bones of this dis ase can be treated by administering a GH-RH antagonist.

Further diseases involving GH are diabetic r tinopathy and diabetic nephropathy. The damage to the retina and kidneys respectively in these diseases, believed to be due to GH, results in blindness or reduction in kidney function. This damage however can be prevented or slowed by administration of an effective GH-RH antagonist.

In an effort to intervene in these disease and other conditions, some investigators have attempted to control GH levels by using somatostatin, one inhibitor of GH release. However, somatostatin, if administered alone, does not suppress GH or IGF-I levels to a desired degree. If administered in combination with a GH-RH antagonist, somatostatin would improve suppression of IGF-I levels much better.

Other workers have investigated various modifications of GH-RH to elucidate the relationship of the structure of GH-RH to its activity in an effort to provide synthetic congeners with improved agonistic or antagonistic properties. (Synthesis may be by solid phase method, described in US Patent 4,914,189, or in liquid phase, as described in US Patent 4,707,541.) Thus, in one study, it was found that synthesizing GH-RH without its N-terminus residue -- i.e., forming hGH-RH(2-44) -- results in an analogue having GH releasing activity which is only 0.1% that of GH-RH. By contrast, synthesizing a GH-RH analogue without its residues 30 through 44 -- i.e., synthesizing hGH-RH(1-29)NH_2 -- results in an analogue which retains 50% or more of the potency of native hGH-RH. Synthesizing even shorter analogues -- e.g., GH-RH(1-28)NH_2 or GH-RH(1-27)NH_2 -- resulted in substantially lower bioactivity. These results indicate that residues 1 and 29 are important to the bioactivity of GH-RH.

In another study, it was found that acetylating the N-t rminus amino acid residue of GH-RH r replacing it with a D-isom r -- thus forming [Ac-Tyr^1]GH-RH or [D-Tyr^1]GH-RH-- lowers the ability of the analogues to r lease

GH to 2-3% that of GH-RH. These analogues als have less affinity in vitro for GH-RH binding sites. By contrast, acetylation of the alpha amino group of residue 1 in hGH-RH(1-29)NH_2 -- thus forming [AcTyr^1]hGH-RH(1-29)NH_2 -- is found to raise the in vivo potency over that of GH-RH by ten fold or more.

In further studies, it was found that [Ac-Tyr^1,D-Arg^2]hGH-RH(1-29) NH_2 ant-agonizes the activation of rat anterior pituitary adenylate cyclase by hGH-RH(1-29)NH_2. The same peptide was found to block the action of GH-RH on its receptors in the pituitary and hypothalamus, and to inhibit the pulsatile growth hormone secretion.

Several reported modifications to GH-RH have resulted in agonistic activity. US Patent 4,659,693 discloses agonists of hGH-RH(1-29) having the formula: R^1-R^2-Asp-Ala-Ile-Phe-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Gly-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-R^{27}-Ser-Arg-NH_2,wherein R^1 is H, Tyr or His; R^2 may be various residues; and R^{27} is Nle. These agonists are said to stimulate release of growth hormone releasing factor ("GRF") and so to be suitable in pharmaceutical compositions. ("GRF" is merely a synonym for GH-RH, and the latter abbreviation is used hereinafter, despite use of GRF in US 4,659,693 and other publications.)

US Patent 4,914,189 discloses other analogues of GH-RH which are agonists. In these agonists, the N-terminus group Q^1CO-, where Q^1 signifies certain omega or alpha-omega substituted alkyl groups, may be Tyr or des-amino-Tyr; the C-terminus group NH-Q^2, where Q^2 signifies certain lower omega-guanidino-alkyl groups, may be Agm; and R^{27} may be Nle. These analogues are said to be extremely potent stimulants of GH release and to njoy high resistance to in vivo nzymatic degradation du t th omega-guanidino-lower alkyl group at the C-terminus.

Published application WO 91/16923 reviews earlier attempts to alter the secondary structure of hGH-RH by modifying its amino acid sequence. These earlier attempts include: replacing Tyr^1, Ala^2, Asp^3 or Asn^8 with their D-isomers; replacing Ser^9 with Ala to enhance amphilicity of the region; and replacing Asn^8 with L- or D-Ser, D-Arg, Asn, Thr, Gln or D-Lys. Certain of these modifications are said to enhance GH releasing activity. WO 91/16923 also states that replacing Asn^8 with Ala induces an enormous increase in GH releasing activity. The peptides said to have this benefit have the formula: $[R^1,R^2,Ala^8,R^{15}, Nle^{27}]$hGH-RH(1-29)-$NH_2$, where R^1 is Dat or A-R^1, where A is lower acyl or benzyl and R^1 includes Tyr and His; R^2 is Ala, D-Ala or N-Me-D-Ala (N-Methyl-D-Ala); and R^{15} may include Gly, Ala or Aib. One preferred embodiment has $R^{8,9,15}$ as Ala. It is noted that R^8 in this publication is never Asn. Pharmaceutical compositions for enhancing growth are further disclosed.

European Patent Application Serial No. 0 413 839 A, filed August 22, 1989, assigned to the same assignee as the present application, discloses analogues of hGH-RH(1-29)-NH_2 said to have enhanced release of GH. The analogues of this application replace residues 1, 2, 8, 12, 15, 27, 28 and 29 as follows: R^1 may be Tyr or Dat; R^2 may be L or D Ala; R^8 may be Asn or Ser; R^{12} may be L or D isomers of Lys, Arg or Orn; R^{15} may be Gly or Ala; R^{27} may be Nle; R^{28} may be Asp, Asn or Ser; and R^{29} may be Agm. However, residue 6 is never replaced: it is always Phe.

Yet another modification of hGH-RH was disclosed in US Patent 5,183,660, where GH-RH was conjugated with polyethylene glycol derivatives. The resulting conjugate was said to exhibit decreased antigenicity, delay in biological clearance in vivo and physiological activity ov r a longer time.

In several of th s investigations, it was found that variants of the hGH-RH agonistic analogues had antagonistic, rather than agonistic, activity. Thus, in US 4,659,693 (where R^2 may be certain D-Arg residues substituted with alkyl groups), when R^1 is H, the hGH-RH analogues are said to act as antagonists. Similarly, in WO 91/16923, discussed above, if R^2 in the analogues is D-Arg, and R^8, R^9, and R^{15} are substituted as indicated above, antagonistic activity is said to result. These antagonistic peptides are said to be suitable for administration as pharmaceutical compositions to treat conditions associated with excessive levels of GH, e.g., acromegaly.

The antagonistic activity of the hGH-RH analogue "[Ser^9-Ψ[CH_2-NH]-Tyr^{10}]hGH-RH(1-29)" of US Patent 5,084,555 was said to result from the pseudopeptide bond (i.e., a peptide bond reduced to a [CH_2-NH] linkage) between the R^9 and R^{10} residues. (It is noted that although this patent employed the seemingly redundant "Ψ[CH_2-NH]" formula for the pseudo-peptide bond, actually only one such linkage had been introduced into the peptide.) However, the antagonistic properties of [Ser^9-Ψ[CH2-NH]-Tyr^{10}] hGH-RH(1-29) were said to be inferior to a conventional antagonist, [N-Ac-Tyr^1, D-Arg^2]GH-RH(1-29)-NH_2.

SUMMARY OF THE INVENTION

There is provided a novel series of synthetic analogues of hGH-RH(1-29)NH_2. These analogues inhibit the activity of endogenous hGH-RH, and therefore prevent the release of growth hormone. This inhibition is believed to result from replacement of various amino acids and acylation with aromatic or nonpolar acids at the N-terminus of GH-RH(1-29)NH_2. The analogues exhibit prolonged antagonistic duration.

Specifically, the invention relates to peptides comprising the formula:

X-R^1-R^2-R^3-R^4-R^5-R^6-Thr-R^8-S r-Tyr-R^{11}-R^{12}-Val-Leu-R^{15}-Gln-Leu-Ser-R^{19}-R^{20}-R^{21}-Leu-L u-Gln-Asp-Ile-R^{27}-R^{28}-R^{29}

wherein

X is nil, H, Ac, IAc, BrProp, For, Ibu, Nac, 2-Nac, 1- or 2-Npt, 1- or 2-Npr or Aqc,

R^1 is Tyr, His, Glt or Glu,

R^2 is D-Arg, D-Cit, D-Har, D-Lys or D-Orn,

R^3 is Asp, Ala or Gly,

R^4 is Ala or Gly,

R^5 is Ile, Ala or Gly,

R^6 is Phe, Ala, Pro, Tpi, Nal, or Phe(Y), in which Y is F, Cl, Br, NO_2, CH_3 or OCH_3,

R^8 is Asn, Ser, Val, Ile, Ala, Abu, Nle, or Aib,

R^{11} is Arg, D-Arg or Cit,

R^{12} is Lys, D-Lys, Cit or Ala,

R^{15} is Gly, Ala, Abu or Gln,

R^{19} is Ala or Abu,

R^{20} is Arg, D-Arg or Cit,

R^{21} is Lys, D-Lys or Cit,

R^{27} is Met, Nle or Abu,

R^{28} is Ser, Asn, Asp or Abu,

R^{29} is Agm, Arg-NH_2, Arg-OH, Cit-NH_2, Cit-OH, Har-NH_2 or Har-OH,

provided that when R^1 is Glt, X is nil, and when X is H, R^{15} is other than Gly,

and pharmaceutically acceptable acid addition salts thereof.

Among the preferred embodiments are peptides wherein X is H and R^{15} is Abu; or wherein X is Nac, For, or Ibu, R^1 is Tyr or His, R^2 is D-Arg or D-Cit, R^3 is Asp, R^4 is Ala, R^5 is Ile, R^6 is Phe(pCl) or Nal, R^{11} is Arg, R^{12} is Lys, R^{15} is Abu or Ala, R^{19} is Ala or Abu, R^{20} is Arg, R^{21} is Lys, R^{27} is Nle, R^{28} is Ser or Asp, and R^{29} is Agm or Arg-NH_2. Thr very preferr d embodiments hav the formulae:

Nac^0-Tyr-D-Arg^2-Asp-Ala-Ile-Ph (pCl)^6-Thr-Asn-S r-Tyr-Arg-Lys-Val-L u-Abu^{15}-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle^{27}-Ser-Agm ("Peptide 18")

Nac^0-Tyr-D-Arg^2-Asp-Ala-Ile-Nal^6-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu^{15}-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle^{27}-Ser-Agm ("Peptide 32")

Nac^0-Tyr-D-Cit^2-Asp-Ala-Ile-Phe(pCl)^6-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu^{15}-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle^{27}-Ser-Agm ("Peptide 34").

Under well-established convention, these may be abbreviated as follows:

[Nac^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm Peptide 18

[Nac^0,D-Arg^2,Nal^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm Peptide 32

[Nac^0,D-Cit^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm Peptide 34

Four especially preferred embodiments have the formulae:

Nac^0-Tyr-D-Arg^2-Asp-Ala-Ile-Phe(pCl)^6-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu^{15}-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle^{27}-Ser-Arg-NH_2 ("Peptide 1")

Nac^0-His^1-D-Arg^2-Asp-Ala-Ile-Phe(pCl)^6-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu^{15}-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle^{27}-Ser-Arg-NH_2 ("Peptide 5")

Ibu^0-Tyr-D-Arg^2-Asp-Ala-Ile-Phe(pCl)^6-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu^{15}-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle^{27}-Ser-Agm ("Peptide 19").

For^0-Tyr-D-Arg^2-Asp-Ala-Ile-Phe(pCl)^6-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu^{15}-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle^{27}-Ser-Agm ("Peptide 38").

These may be represented by well-accepted convention respectively as follows:

[Nac^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nl ^{27}]hGH-RH(1-29)NH_2 P ptid 1

[Nac^0,His^1-D-Arg^2,Ph (pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH_2 P ptide 5

[Ibu^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm Peptide 19

[For^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm Peptide 38

It is noted that the amino acid residues from 30 through 44 of the native GH-RH molecule do not appear to be essential to activity; nor does their identity appear to be critical. Therefore, it appears that the addition of some or all of these further amino acid residues to the C-terminus of the hGH-RH(1-29)-NH_2 analogues of the present invention will not affect the efficacy of these analogues as GH antagonists. If some or all of these amino acids were added to the C-terminus of the hGH-RH(1-29)-NH_2 analogues, the added amino acid residues could be the same as residues 30 through 44 in the native hGH-RH sequence or reasonable equivalents.

Synthetic Methods.

The synthetic peptides are synthesized by a suitable method such as by exclusive solid phase techniques, by partial solid-phase techniques, by fragment condensation or by classical solution phase synthesis.

When the analogues of this invention are synthesized by solid-phase method, the C-terminus residue (here, R^{29}) is appropriately linked (anchored) to an inert solid support (resin) while bearing protecting groups for its alpha amino group (and, where appropriate, for its side chain functional group). After completion of this step, the alpha amino protecting group is removed from the anchored amino acid residue and the next amino acid residue, R^{28}, is added having its alpha amino group (as well as any appropriate side chain functional group) suitably protected, and so forth. The N-terminus protecting groups are removed after each residue is added, but the side chain protecting groups are not yet removed. After all the desired amino acids have been linked in the prop r sequence, the peptide is cleaved from the support and freed from any side chain pr tecting group(s) und r conditions that are minimally destructive towards residues in the sequence.

This is be followed by a careful purification and scrupulous characterization of the synthetic product, so as to ensure that the desired structure is indeed the one obtained.

It is particularly preferred to protect the alpha amino function of the amino acids during the coupling step with an acid or base sensitive protecting group. Such protecting groups should have the properties of being stable in the conditions of peptide linkage formation, while being readily removable without destruction of the growing peptide chain and without racemization of any of the chiral centers contained therein. Suitable alpha amino protecting groups are Boc and Fmoc.

Medical Applications.

The hGH-RH antagonist peptides, or salts of these peptides, may be formulated in pharmaceutical dosage forms containing effective amounts thereof and administered to humans or animal for therapeutic or diagnostic purposes. The peptides may be used to suppress GH levels and to treat conditions associated with excessive levels of GH, e.g., diabetic retinopathy and nephropathy, and acromegaly. Also provided are methods for treating these diseases by administration of a composition of the invention to an individual needing such treatment. The main uses of GH-RH antagonists are however,in the field of cancer, for example human cancers of the breast, lung, colon, brain, and pancreas where the receptors for IGF-I are present.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a plot of tumor volumes in athymic nude mice bearing s.c. transplanted SK-ES-1 human sarcomas during treatment with Peptide 19 administered from osmotic minipumps at a dose of 40μg/animal/day. Treatment was started when the tumors measured approximately 33-39 mm^3 and lasted for 4 and 3 weeks, respectively.

Figure 2 is a plot of tumor volumes in athymic nud mice b aring s.c. transplanted MNNG/HOS human sarc mas during treatment with Peptide 19 administered from osmotic minipumps at a dose of 40μg/animal/day.

Treatment was started when the tumors measured approximately 33-39 mm^3 and lasted for 4 and 3 weeks, respectively.

Figure 3 is a plot of the inhibitory effect of GH-RH antagonist Peptide 19 on growth of MXT estrogen independent mouse mammary cancer.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

A. Abbreviations

The nomenclature used to define the peptides is that specified by the IUPAC-IUB Commissioner on Biochemical Nomenclature wherein, in accordance with conventional representation, the amino group at the N-terminus appears to the left and the carboxyl group at the C-terminus appears to the right. The term "natural amino acid" as used herein means one of the common, naturally occurring L-amino acids found in naturally occurring proteins: Gly, Ala, Val, Leu, Ile, Ser, Thr, Lys, Arg, Asp, Asn, Glu, Gln, Cys, Met Phe, Tyr, Pro, Trp and His. When the natural amino acid residue has isomeric forms, it is the L-form of the amino acid that is represented herein unless otherwise expressly indicated.

Non-coded amino acids, or amino acid analogues, are also incorporated into the GH-RH antagonists. ("Non-coded" amino acids are those amino acids which are not among the approximately 20 natural amino acids found in naturally occurring peptides.) Among the non-coded amino acids or amino acid analogues which may be used in the hGH-RH antagonist peptides are the following: by Abu is meant alpha amino butyric acid, by Agm is meant agmatine (1-amino-4-guanidino-butane), by Aib meant alpha amino isobutyric acid, by Har is meant homoarginine, by hPhe is meant homo-phenylalanine, by Nal is meant 2-naphthyl-alanine, and by Nle is meant norleucine. When these non-coded amino acids, or amino acid analogues, have isomeric forms, it is the L-form of the amino acid that is represented unless otherwise expressly indicated.

Abbreviations used herein are:

Abu	α-aminobutyric acid
Ac	acetyl
AcOH	acetic acid
Ac_2O	acetic anhydride
Agm	agmatine (1-amino-4-guanidino-butane)
Aib	α-aminoisobutyric acid
Aqc	anthraquinone-2-carbonyl
BHA	benzhydrylamine
Boc	tert.butyloxycarbonyl
Bom	benzyloxymethyl
BOP	benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate
BrProp	bromopropionyl
Bzl	benzyl
cHx	cyclohexyl
Cit	citrulline, i.e., 2-amino-5-ureidovaleric acid
DCC	dicyclohexylcarbodiimide
DCM	dichloromethane
DIC	N,N'-diisopropylcarbodiimide
DIEA	diisopropylethylamine
DMF	dimethylformamide
Fmoc	fluorenylmethyloxycarbonyl
For	Formyl
GH	growth hormone
GH-RH	GH releasing hormone
Glt	glutaryl
Har	homoarginine
hGH-RH	human GH-RH
HOBt	1-hydroxybenzotriazole
hPhe	homoph nylalanine

HPLC	high performance liquid chromatography
IAc	iodoacetyl
Ibu	isobutyryl
MeOH	methanol
MeCN	acetonitrile
MBHA	para-methylbenzhydrylamine
Nac	1-naphthylacetyl
2-Nac	2-naphthylacetyl
Nal	2-naphthyl-alanine
Nle	norleucine
NMM	N-methylmorpholine
Npr	naphthylpropionyl
1-Npt	1-naphthoyl
2-Npt	2-naphthoyl
Phe(pCl)	para-chloro-phenylalanine
rGH-RH	rat GH-RH
RP-HPLC	reversed phase HPLC
SPA	sulfophenoxy acetyl
TFA	trifluoroacetic acid
Tos	para-toluenesulfonyl
Tpi	2,3,4,9-tetrahydro-1H-pyrido[3,4-b]indol-3-carboxylic acid
Z	benzyloxycarbonyl
ϕ	unsubstituted aromatic ring

B. The GH-RH Analogues

The hGH-RH analogues of the present invention were designed to increase the affinities of the peptides to the receptor, to improve metabolic stability and to maximize the amphophilic secondary structur of th molecules. Many of these analogu s cause v ry ffective and l ng lasting inhibition of GH release stimulated by hGH-RH(1-29)NH_2.

The following embodiments are specially preferred as having remarkable bioactivity:

[Nac^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH$_2$	Peptide # 1
[Ac^0-His^1,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH$_2$	Peptide # 2
[Ibu^0-His^1,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH$_2$	Peptide # 3
[IAc^0-His^1,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH$_2$	Peptide # 4
[Nac^0-His^1,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH$_2$	Peptide # 5
[Glt^1,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH$_2$	Peptide # 6
[Ibu^0-Glu^1,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH$_2$	Peptide # 7
[IAc^0-Glu^1,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH$_2$	Peptide # 8
[Nac^0-Glu^1,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH$_2$	Peptide # 9
[Ibu^0-His^1,D-Arg^2,Tpi^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH$_2$	Peptide # 10
[IAc^0-His^1,D-Arg^2,Tpi^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH2	Peptide # 11
[Glt^1,D-Arg^2,Tpi^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH2	Peptide # 12
[Ibu^0,D-Arg^2,Aib^8,Abu^{15},Nle^{27}]hGH-RH(1-29)NH2	Peptide # 13
[Ibu^0,D-Arg^2,Phe(pCl)^6,Aib^8,Abu^{15},Nle^{27}]hGH-RH(1-29)NH2	Peptide # 14
[Ibu^0,D-Arg^2,Phe(pCl)^6,Ala^{12},Abu^{15},Nle^{27}]hGH-RH(1-29)NH2	Peptide # 15
[Ibu^0,D-Arg^2,Phe(pCl)^6,Abu^{15,19},Nle^{27}]hGH-RH(1-29)NH2	Peptide # 16
[Ibu^0-Glu^1,D-Arg^2,Phe(pCl)^6,Abu^{15,19},Nle^{27}]hGH-RH(1-29)NH2	Peptide # 17
[Nac^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm	Peptide # 18
[Ibu^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm	Peptide # 19
[BrProp^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm	Peptide # 20
[IAc^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm	Peptide # 21
[Nac^0-His^1,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27},Asp^{28}]hGH-RH(1-28)Agm	Peptide # 22
[Nac^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27},Asp^{28}]hGH-RH(1-28)Agm	Peptide # 23
[2-Nac^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27},Asp^{28}]hGH-RH(1-28)Agm	Peptide # 24
[1-Npt^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27},Asp^{28}]hGH-RH(1-28)Agm	Peptide # 25
[Aqc^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27},Asp^{28}]hGH-RH(1-28)Agm	Peptide # 26
[Nac^0,D-Arg^2,Phe(pCl)^6,Ala^{15},Nle^{27}]hGH-RH(1-28)Agm	Peptide # 27
[Nac^0,D-Arg^2,Gly^3,Phe(pCl)^6,Abu^{15},Nle^{27},Asp^{28}]hGH-RH (1-28)Agm	Peptide # 28
[IAc^0,D-Arg^2,Pro^6,Abu^{15},Nle^{27},Asp^{28}]hGH-RH(1-28)Agm	Peptide # 29
[Ibu^0,D-Arg^2,Pro^6,Abu^{15},Nle^{27},Asp^{28}]hGH-RH(1-28)Agm	Peptide # 30
[IAc^0,D-Arg^2,hPhe^6,Abu^{15},Nle^{27},Asp^{28}]hGH-RH(1-28)Agm	Peptide # 31

[Nac^0,D-Arg^2,Nal^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm Peptide # 32

[Nac^0,D-Arg^2,Ala^6,Abu^{15},Nle^{27},Asp^{28}]hGH-RH(1-28)Agm Peptide # 33

[Nac^0,D-Cit^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm Peptide # 34

[D-Cit^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm Peptide # 35

[Nac^0,D-Cit^2,Nal^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm Peptide # 36

[D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm Peptide # 37

[For^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm Peptide # 38

Three highly preferred embodiments have the following formulae:

[Nac^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm Peptide # 18

[Nac^0,D-Arg^2,Nal^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm Peptide # 32

[Nac^0,D-Cit^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm Peptide # 34

The most preferred embodiments have the following formulae:

[Nac^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH_2 Peptide # 1

[Nac^0-His^1-D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH_2 Peptide # 5

[Ibu^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm Peptide # 19

[For^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm Peptide # 38

C. Method of Preparation

1. Overview of Synthesis.

The peptides are synthesized by a suitable method such as by exclusive solid phase techniques, by partial solid-phase techniques, by fragment condensation or by classical solution phase synthesis. For example, the techniques of exclusive solid-phase synthesis are set forth in the textbook "Solid Phase Peptide Synthesis", J.M. Stewart and J.D. Young, Pierce Chem. Company, Rockford, 111, 1984 (2nd. ed.), and M. Bodanszky, "Principles of Peptide Synthesis", SpringerVerlag, 1984. The hGH-RH antagonist peptides are preferably prepared using solid phase synthesis, such as that generally described by Merrifield, J.Am.Chem.Soc., 85, p. 2149 (1963), although oth r quivalent chemical synth s s known in the art can also be used as previously mentioned.

Th synth sis is carried out with amino acids that ar prot cted at their alpha amino group. Urethane type protecting groups (Boc or Fmoc) are preferably used for the protection of the alpha amino group. The preferred protecting group is Boc.

In solid phase synthesis, the moiety which forms the aminoacyl group of the final peptide at the C-terminus is attached to a polymeric resin support via a chemical link. After completion of the coupling reaction, the alpha amino protecting group is selectively removed to allow subsequent coupling reactions to take place at the amino-terminus, preferably with 50% TFA in DCM. The remaining amino acids with similarly Boc-protected alpha amino groups are coupled stepwise to the free amino group of the preceding amino acid on the resin to obtain the desired peptide sequence. Because the amino acid residues are added to the alpha amino group of the C-terminus residue, growth of the synthetic hGH-RH analogue peptides begins at the C terminus and progresses toward the N-terminus. When the desired sequence has been obtained, the peptide is acylated, if appropriate, and it is removed from the support polymer.

Each protected amino acid is used in excess (2.5 or 3 equivalents) and the coupling reactions are usually carried out in DCM, DMF or mixtures thereof. The extent of completion of the coupling reaction is monitored at each stage by the ninhydrin reaction. In cases where incomplete coupling is determined, the coupling procedure is repeated before removal of the alpha amino protecting group prior to the coupling of the next amino acid.

A typical synthesis cycle is shown in Table I.

TABLE I

Protocol for a Typical Synthetic Cycle Using Boc-strategy

Step	Reagent	Mixing Time (min)
1. Deprotection 5+25	50% TFA in DCM	
	DCM wash	1
	2-propanol wash	1
2. Neutralization	5% DIEA in DCM	1
	DCM wash	1
	MeOH wash	1
	5% DIEA in DCM	3
	MeOH wash	1
	DCM wash (3 times)	1-1
3. Coupling	3 equiv. Boc-amino acid in DCM or DMF + 3 equiv. DIC or the preformed HOBt ester of the Boc-amino acid	60
	MeOH wash	2
	DCM wash	2
	MeOH wash	2
	DCM wash	2
	MeOH wash	2
	DCM wash	2
4. Acetylation + 20 (if appropriate)	Ac_2O in DCM (30%)	10
	MeOH wash (3 times)	2
	DCM wash (3 times)	2

After completion of the synthesis, the cleavage of the peptide from the resin can be effected using procedures well known in peptide chemistry.

Some of the amino acid residues of th peptides hav side chain functional groups which are reactive with reagents used in coupling or deprotection. When such side chain groups are present, suitable protecting groups are joined to these functional groups to prevent undesirable chemical reactions from occurring during the reactions used to form the peptides. The following general rules are followed in selecting a particular side chain protecting group: (a) the protecting group preferably retains its protecting properties and is not split off under coupling conditions, (b) the protecting group should be stable to the reagent used in the coupling reaction conditions and in conditions for removing the alpha amino protecting group at each step of the synthesis and, (c) the side chain protecting group must be removable upon the completion of the synthesis of the desired amino acid sequence, under reaction conditions that will not undesirably alter the peptide chain.

The initial synthetic steps utilized herein are disclosed in US Patent 4,914,189 which is incorporated by reference herein. Reference is particularly made to Examples I through IV therein.

2. Coupling R^{29} to the Support Polymer.

The hGH-RH antagonist peptides may be synthesized on a variety of support polymers. These support polymers may be amino resins such as amino-methyl resins, benzhydrylamine resins, p-methylbenzhydrylamine resins and the like. Boc-R^{29} is the initial material joined to the support phase, suitably Boc-Arg(Tos)-OH or Boc-Agm.

For the synthesis of peptides having Agm at the C-terminus, it is preferred that the support phase [SP] is an amino methyl resin. The guanidin group of Boc-Agm is joined to th support polym r via a stable but readily cleavable bridging group. It has been found that such a bridge may be r adily provided by th sulfonyl ph noxy acetyl moi ty. The alpha

amino Boc-protected Agm is reacted with the chlorosulfonyl phenoxy acetic acid

$$Cl\text{-}SO_2\text{-}\phi\text{-}O\text{-}CH_2\text{-}COOH$$

to form

$$Boc\text{-}Agm^{29}\text{-}SO_2\text{-}\phi\text{-}O\text{-}CH_2\text{-}COOH.$$

This compound is then coupled to the support polymer [SP] using DIC or BOP as activating reagent to yield:

$$Boc\text{-}Agm^{29}\text{-}SO_2\text{-}\phi\text{-}O\text{-}CH_2\text{-}CO\text{-}[SP]$$

For the synthesis of peptides having Arg-NH_2 at the C-terminus, Boc-Arg(Tos)-OH is coupled to the neutralized BHA or MBHA resin using DIC or BOP as activating reagent.

3. Stepwise Coupling of Amino Acid Residues.

Utilizing the Boc-protected Agm resin (California Peptide Res. Inc.), (or the Boc-Arg(Tos)-resin), the peptide itself may suitably be built up by solid phase synthesis in the conventional manner. The selection of an appropriate coupling reagent is within the skill of the art. Particularly suitable as coupling reagents are N,N'-diisopropyl carbodiimide (DIC) or the BOP carboxyl activating reagent.

Each protected amino acid is coupled in about a three-fold molar excess, with respect to resin-bound aminoacyl residue(s), and the coupling may be carried out in as medium such as DMF: CH_2Cl_2 (1:1) or in DMF or CH_2Cl_2 alone. In cases where incomplete coupling occurs, the coupling procedure is repeated before removal of the alpha amino protecting group. The success of the coupling reaction at each stage of the synthesis is pref rably monitored by the ninhydrin reaction.

4. Removal of th Peptide from the Support Polymer.

When the synthesis is complete, the peptide is cleaved from the support phase. Removal of the peptide from the resin is performed by treatment with a reagent such as liquid hydrogen fluoride which also cleaves all remaining side chain protecting groups.

Suitably, the dried and protected peptide-resin is treated with a mixture consisting of 1.0 mL m-cresol and 10 mL anhydrous hydrogen fluoride per gram of peptide-resin for 60 min at 0°C to cleave the peptide from the resin as well as to remove all side chain protecting groups. After the removal of the hydrogen fluoride under a stream of nitrogen and vacuum, the free peptides are precipitated with ether, filtered, washed with ether and ethyl acetate, extracted with 50% acetic acid, and lyophilized.

5. Purification

The purification of the crude peptides can be effected using procedures well known in peptide chemistry. For example, purification may be performed on a MacRabbit HPLC system (Rainin Instrument Co. Inc., Woburn, MA) with a Knauer UV Photometer and a Kipp and Zonen BD40 Recorder using a 10 x 250 mm VYDAC 228TP column packed with C8 silica gel (300 Å pore size, 10 μm particle size) (Rainin Inc.). The column is eluted with a solvent system consisting of (A) 0.1% aqueous TFA and (B) 0.1% TFA in 70% aqueous MeCN in a linear gradient mode (e.g., 30-65% B in 120 min). The eluent is monitored at 220 nm, and fractions are examined by analytical HPLC using a Hewlett-Packard Model HP-1090 liquid chromatograph and pooled to give maximum purity. Analytical HPLC is carried out on a W-Porex C18 reversed-phase column (4.6 x 250 mm, 5 μm particle size, 300 Å pore size) (Phenomenex, Rancho Palos Verdes, CA) using is cratic elution with a solv nt system consisting of (A) and (B) defined above. Th peaks are monitored at 220 and 280 nm. Th peptides

are judged to be substantially (>95%) pure by analytical HPLC. The expected amino acid composition is also confirmed by amino acid analysis.

D. Pharmaceutical Composition

The peptides of the invention may be administered in the form of pharmaceutically acceptable, nontoxic salts, such as acid addition salts. Illustrative of such acid addition salts are hydrochloride, hydrobromide, sulphate, phosphate, fumarate, gluconate, tannate, maleate, acetate, citrate, benzoate, succinate, alginate, pamoate, malate, ascorbate, tartarate, and the like. Particularly preferred antagonists are salts of low solubility, e.g., pamoate salts and the like. These exhibit long duration of activity.

The compounds of the present invention are suitably administered to subject humans or animals s.c., i.m., or i.v; intranasally or by pulmonary inhalation; or in a depot form (e.g., microcapsules, microgranules, or cylindrical rod like implants) formulated from a biodegradable suitable polymer (such as D,L-lactide-coglycolide), the former two depot modes being preferred. Other equivalent modes of administration are also within the scope of this invention, i.e., continuous drip, depot injections, infusion pump and time release modes such as microcapsules and the like. Administration is in any physiologically acceptable injectable carrier, physiological saline being acceptable, though other carriers known to the art may also be used.

The peptides are preferably administered parenterally, intramuscularly, subcutaneously or intravenously with a pharmaceutically acceptable carrier such as isotonic saline. Alternatively, the peptides may be administered as an intranasal spray with an appropriate carrier or by pulmonary inhalation. One suitable route f administration is a depot form formulated from a biodegradable suitable polymer, e.g., poly-D,L-lactide-coglycolide as

microcapsules, microgranules or cylindrical implants containing dispersed antagonistic compounds.

The amount of peptide needed depends on the mode of administration and the intended result. In general, the dosage range is between 1-100 μg/kg of body weight of the host per day.

E. Therapeutic Uses of GH-RH Antagonists

hGH-RH antagonists can be used in treatment of conditions caused by excess growth hormone, for example acromegaly, which is manifested by an abnormal enlargement of the bones of the face and extremities. The GH-RH antagonists may also be used to treat diabetic retinopathy (the main cause of blindness in diabetics) and diabetic retinopathy, in which damage to the eye and kidney respectively is thought to be due to GH.

The hGH-RH antagonists are designed to block the binding and therefore the action of GH-RH, which stimulates the secretion of GH, which in turn stimulates production of IGF I. GH-RH antagonists may be administered alone or together with somatostatin analogues, a combination which more completely suppresses IGF-I levels. It is advantageous to administer antagonists of GH-RH rather than somatostatin due to the fact that GH-RH antagonists may be utilized in situations where target sites do not have somatostatin receptors.

However, the main applications of GH-RH antagonists are in the field of cancer. This is based on the following considerations: GH-RH antagonists are designed to block the binding and therefore the action of GH-RH, which stimulates the secretion of GH, which in turn stimulates production of insulin-like growth factor I(IGF-I) also called somatomedin-C. The involvement of IGF-I (somatomedin-C) in breast cancer, prostate cancer, colon cancer, bone tumors and other malignancies is well established, and

somatostatin analogues alone do not adequately suppress GH and IGF-I levels. A complete suppression of IGF-I levels or secretion is required for a better inhibition of tumor growth. Autocrine production of IGF-I by various tumors could be also under control of GH-RH and might therefore be inhibited by GH-RH antagonists. GH-RH antagonists might also inhibit the production of IGF-I. A more detailed theoretical background of the applications of GH-RH in the field of oncology (cancer) is as follows: The receptors for IGF-I are present in primary human breast cancers, in lung cancers, in human colon cancers, in human brain tumors, and in human pancreatic cancers.

The presence of IGF-I receptors in these tumors appears to be related to malignant transformation and proliferations of these cancers. IGF-I can act as endocrine, paracrine or autocrine growth factor for various human cancers, that is the growth of these neoplasms is dependent on IGF-I. GH-RH antagonists by suppressing GH secretion would lower the production of IGF-I. Since IGF-I stimulates growth of these various neoplasms (cancers), the lowering of circulating IGF-I levels should lead to tumor growth inhibition. It is possible that GH-RH antagonists could also lower paracrine or autocrine production of IGF-I by the tumors, which should also lead to inhibition of cancer proliferation. These views are in accordance with modern concepts of clinical oncology. GH-RH antagonists should be given alone or together with somatostatin analogues and a combination would achieve a more complete suppression of IGF-I levels, elimination of tissue IGF-I levels, e.g., in human osteosarcomas, as well as breast cancer, colon cancer, prostate cancer, and non-small cell lung cancer (non-SCLC).

The advantage of GH-RH antagonists over somatostatin analogues is based n the fact that GH-RH antagonists may be utilized for suppressi n of tumors which do not have somatostatin receptors, f r example human osteogenic sarc mas.

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The present invention is described in connection with the following examples which are set forth for the purposes of illustration only.

The following Examples set forth suitable methods of synthesizing the novel GH-RH antagonists by the solid-phase technique.

EXAMPLE I

Synthesis of Boc-agmatine

EXAMPLE II

Synthesis of 4-Chlorosulfonyl Phenoxyacetic Acid (Cl-SPA)

EXAMPLE III

Boc-agmatine-[SPA]

EXAMPLE IV

Coupling of Boc-agmatine-[SPA] to Support Phase

The initial synthetic sequence utilized herein and indicated by headings above is disclosed in Examples I through IV of US Patent 4,914,189, which Examples are incorporated herein by reference.

EXAMPLE V

The synthesis of Peptide 1 having the formula:

Nac^{0}-Tyr^{1}-D-Arg^{2}-Asp^{3}-Ala^{4}-Ile^{5}-$Phe(pCl)^{6}$-Thr^{7}-Asn^{8}-Ser^{9}-Tyr^{10}-Arg^{11}-Lys^{12}-Val^{13}-Leu^{14}-Abu^{15}-Gln^{16}-Leu^{17}-Ser^{18}-Ala^{19}-Arg^{20}-Lys^{21}-Leu^{22}-Leu^{23}-Gln^{24}-Asp^{25}-Ile^{26}-Nle^{27}-Ser^{28}-Arg^{29}-NH_2

or $[Nac^{0}$,D-Arg^{2},$Phe(pCl)^{6}$,Abu^{15},$Nle^{27}]$hGH-RH(1-29)NH_2

is conducted in a stepwise manner using manual solid phase peptide synthesis equipment. Briefly, 4-methyl-benzhydrylamine (MBHA) resin (Bachem, California) (200 mg, 0.11 mmole) is neutralized with 5% DIEA in CH_2Cl_2 and washed according to the protocol described in Table I. The solution of Boc-Arg(Tos)-OH (141 mg, 0.33 mmole) in DMF-CH_2Cl_2 (1:1) is shaken with the neutralizer and DIC (57 μL, 0.36 mmole) in a manual solid phase peptide synthesis equipment for 1 hour. After the completion

of the coupling reaction is proved by negative ninhydrin test, deprotection with 50% TFA in CH_2Cl_2, and neutralization with 5% DIEA, the peptide chain is built stepwise by adding the following protected amino acids in the indicated order on the resin to obtain the desired peptide sequence:

Boc-Ser(Bzl)-OH, Boc-Nle-OH, Boc-Ile-OH, Boc-Asp(OcHx)-OH, Boc-Gln-OH, Boc-Leu-OH, Boc-Leu-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Arg(Tos)OH, Boc-Ala-OH, Boc-Ser(Bzl)-OH, Boc-Leu-OH, Boc-Gln-OH, Boc-Abu-OH, Boc-Leu-OH, Boc-Val-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Arg(Tos)OH, Boc-Tyr(2,6-diCl-Z)-OH, Boc-Ser(Bzl)-OH, Boc-Asn-OH, Boc-Thr(Bzl)-OH, Boc-Phe(pCl)-OH, Boc-Ile-OH, Boc-Ala-OH, Boc-Asp(OcHx)-OH, Boc-D-Arg(Tos)OH, and Boc-Tyr(2,6-diCl-Z)-OH.

These protected amino acid residues (also commonly available from Bachem Co.) are represented above according to a well accepted convention. The suitable protecting group for the side chain functional group of particular amino acids appears in parentheses. The OH groups in the above formulae indicate that each residue's carboxyl terminus is free.

The protected amino acids (0.33 mmole each) are coupled with DIC (57 μL, 0.36 mmole), with the exceptions of Boc-Asn-OH and Boc-Gln-OH which are coupled with their preformed HOBt esters. After removal of the Boc protecting group from the alpha amino group of Tyr^1, the alpha amino group of Tyr^1 is acylated. This is performed by the symmetrical anhydride method, in which 1-naphthylacetic acid (123 mg, 0.66 mmole) is reacted with DIC as an activating agent (60 μl, 0.37 mmole) to form a symmetric anhydride of 1-naphthylacetic acid. This symmetrical anhydride is reacted with the peptide.

In order to cleave the peptide from the resin and deprotect it, the dried peptide resin (325 mg) is stirred with 0.5 mL m-cresol and 5 mL hydrogen fluoride (HF) at 0°C for 1 hour. After vaporation of the HF under

vacuum, the r mnant is wash d with dry diethyl eth r and thyl acetat . The cleaved and deprotected peptide is dissolved in 50 % acetic acid and separated from the resin by filtration. After dilution with water and lyophilization, 145 mg crude product is obtained.

The crude peptide is checked by analytical HPLC using a Hewlett-Packard Model HP-1090 liquid chromatograph with a W-Porex C18 reversed-phase column (4.6 x 250mm, 5μm particle size, 300 Å pore size from Phenomenex, Rancho Palos Verdes, CA) and linear gradient elution, (e.g., 35-70% B) with a solvent system consisting of (A) 0.1% aqueous TFA and (B) 0.1% TFA in 70% aqueous MeCN. 60 mg of the crude peptide is dissolved in AcOH/H_2O), stirred, filtered and applied on a VYDAC 228TP column (10 x 250 mm) packed with C8 silica gel. The column is eluted with a solvent system described above in a linear gradient mode (e.g., 30-55% B in 120 min); flow rate 3mL/min. The eluent is monitored at 220 nm, and fractions are examined by analytical HPLC. Fractions with purity higher than 95% are pooled and lyophilized to give 3.5 mg pure product. The analytical HPLC is carried out on a W-Porex C18 reversed-phase column described above using isocratic elution with a solvent system described above with a flow rate of 1.2 mL/min. The peaks are monitored at 220 and 280 nm. R_t = 13.70 min and k' = 0.828 (isocratic elution with 52% B). The peptides are judged to be substantially (>95%) pure by analytical HPLC. The expected amino acid composition is also confirmed by amino acid analysis.

Peptides 2, 3, 4 and 5 are synthesized in the same manner as Peptide 1, except that Boc-Tyr(2,6-diCl-Z)-OH[1] is replaced with Boc-His(Bom)-OH[1] (0.33 mmole) and the resulting peptides are acylated with the appropriate anhydrid s of acetic acid, isobutyric acid, iodoacetic acid or 1-naphthyl-acetic acid r spectively, to yield:

[Ac^0-His^1,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH_2 Peptide 2

[Ibu^0-His^1,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH2 Peptide 3

[IAc^0-His^1,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH_2 Peptide 4

[Nac^0-His^1,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH_2 Peptide 5

Peptide 6 is synthesized in the same manner as Peptide 1, except that Boc-Tyr(2,6-diCl-Z)-OH^1 is omitted, and the final peptide's N-terminus D-Arg is acylated with glutaric anhydride to yield:
[Glt^1,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH_2.

Peptides 7, 8 and 9 are synthesized in the same manner as Peptide 1 except that Boc-Tyr(2,6-diCl-Z)-OH^1 is replaced by Boc-Glu(OcHx)-OH^1 (0.33 mmole) and is acylated with the appropriate anhydride of isobutyric acid, iodoacetic acid and 1-naphthylacetic acid respectively, to yield:

[Ibu^0-Glu^1,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH_2 Peptide 7

[IAc^0-Glu^1,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH_2 Peptide 8

[Nac^0-Glu^1,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH_2 Peptide 9

EXAMPLE VI

The synthesis of Peptide 10 having the formula:
Ibu^0-His^1-D-Arg^2-Asp^3-Ala^4-Ile^5-Tpi^6-Thr^7-Asn^8-Ser^9-Tyr^{10}-Arg^{11}-Lys^{12}-Val^{13}-Leu^{14}-Abu^{15}-Gln^{16}-Leu^{17}-Ser^{18}-Ala^{19}-Arg^{20}-Lys^{21}-Leu^{22}-Leu^{23}-Gln^{24}-Asp^{25}-Ile^{26}-Nle^{27}-Ser^{28}-Arg^{29}-NH_2
or [Ibu^0-His^1,D-Arg^2,Tpi^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH_2 is conducted in a stepwise manner using manual solid phase peptide synthesis equipment. Benzhydrylamine (BHA) resin (Bachem, California) (200 mg, 0.11 mmole) is neutralized with 5 % DIEA in CH_2Cl_2 and washed according to the protocol described in Table I. The solution of Boc-Arg(Tos)-OH (141 mg, 0.33 mmole) in CH_2Cl_2-DMF (1:1) is shak n with the neutralized resin and DIC (60 μL, 0.37 mmole) in a manual solid phase peptide synthesis equipment for 1 hour. After the coupling reaction is proved to b complete

by negative ninhydrin test, deprotection with 50% TFA in CH_2Cl_2, and neutralization with 5% DIEA in CH_2Cl_2, the peptide chain is built by stepwise addition of the following protected amino acids in the indicated order on the resin to obtain the desired peptide sequence:

Boc-Ser(Bzl)-OH, Boc-Nle-OH, Boc-Ile-OH, Boc-Asp(OcHx)-OH, Boc-Gln-OH, Boc-Leu-OH, Boc-Leu-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Arg(Tos)OH, Boc-Ala-OH, Boc-Ser(Bzl)-OH, Boc-Leu-OH, Boc-Gln-OH, Boc-Abu-OH, Boc-Leu-OH, Boc-Val-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Arg(Tos)OH, Boc-Tyr(2,6-diCl-Z)-OH, Boc-Ser(Bzl)-OH, Boc-Asn-OH, Boc-Thr(Bzl)-OH, Boc-Tpi-OH, Boc-Ile-OH, Boc-Ala-OH, Boc-Asp(OcHx)-OH, Boc-D-Arg(Tos)OH, and Boc-His(Bom)-OH.

The protected amino acids (0.33 mmole each) are coupled with DIC (57 μL, 0.36 mmole) with the exceptions of Boc-Asn-OH and Boc-Gln-OH which are coupled with their preformed HOBt esters and Boc-Tpi-OH which was coupled by using BOP coupling method. After removal of the Boc protecting group from the alpha amino group of His^1, the peptide is acylated using the symmetrical anhydride method. This is performed by reacting isobutyric acid (59 mg, 0.66 mmole) with DIC (60μl, 0.37 mmole) to form the symmetrical anhydride thereof, and reacting this anhydride with the peptide.

In order to cleave the peptide from the resin and deprotect it, the dried peptide resin (300-350 mg) is stirred with 0.5 mL m-cresol and 5 mL hydrogen fluoride (HF) at 0°C for 1 hour. After evaporation of the HF under vacuum, the remnant is washed with dry diethyl ether and ethyl acetate. The cleaved and deprotected peptide is dissolved in 50% acetic acid and separated from the resin by filtration. After dilution with water and lyophilization, approximately 150 mg crude product is obtained.

The crude peptide is purified (60 mg of the substance being purified by RP-HPLC using the same procedure and equipments described in Example

V), then checked by analytical HPLC. The product is judg d to be substantially (>95%) pure by analytical HPLC. Confirmation of the structure is provided by amino acid analysis.

Peptide 11 is synthesized in the same manner as Peptide 10, except it is acylated with the appropriate anhydride of iodoacetic acid in place of isobutyric acid, to yield:
[IAc^0-His^1,D-Arg^2,Tpi^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH_2.

Peptide 12 is synthesized in the same manner as Peptide 10 except that Boc-His(Bom)-OH^1 and Ibu^0 are omitted. The final peptide's N-terminus D-Arg is acylated with glutaric anhydride to yield:
[Glt^1,D-Arg^2,Tpi^6,Abu^{15}, Nle^{27}]hGH-RH(1-29)NH_2.

Peptide 13 is synthesized in the same manner as Peptide 10 except that Boc-His(Bom)-OH^1 is replaced with Boc-Tyr(2,6-diCl-Z)-OH^1; Boc-Tpi-OH^6 is replaced with Boc-Phe-OH^6; and Boc-Asn-OH^8 is replaced with Boc-Aib-OH^8, to yield:
[Ibu^0,D-Arg^2,Aib^8,Abu^{15},Nle^{27}]hGH-RH(1-29)NH_2.

Peptide 14 is synthesized in the same manner as Peptide 13, except that Boc-Phe-OH^6 is replaced with Boc-Phe(pCl)-OH^6 to yield:
[Ibu^0,D-Arg^2,Phe(pCl)^6,Aib^8,Abu^{15},Nle^{27}]hGH-RH(1-29)NH_2.

Peptide 15 is synthesized in the same manner as Peptide 14 except that Boc-Aib-OH^8 is replaced with Boc-Asn-OH^8 and Boc-Lys(2-Cl-Z)-OH^{12} is replaced with Boc-Ala-OH^{12} to yield:
[Ibu^0,D-Arg^2,Phe(pCl)^6,Ala^{12}, Abu^{15},Nle^{27}]hGH-RH(1-29)NH_2.

Peptide 16 is synthesized in the same manner as Peptide 15 except that Boc-Ala-OH[12] is replaced with Boc-Lys(2-Cl-Z)-OH[12] and Boc-Ala[19]-OH is replaced with Boc-Abu-OH to yield:
[Ibu[0],D-Arg[2],Phe(pCl)[6],Abu[15,19], Nle[27]]hGH-RH(1-29)NH_2.

Peptide 17 is synthesized in the same manner as Peptide 16 except that Boc-Tyr(2,6-diCl-Z)-OH[1] is replaced with Boc-Glu(OcHx)-OH[1], to yield:
[Ibu[0]-Glu[1],D-Arg[2],Phe(pCl)[6],Abu[15,19],Nle[27]]hGH-RH(1-29)NH_2.

EXAMPLE VII

The synthesis of Peptide 18 having the formula:
Nac[0]-Tyr[1]-D-Arg[2]-Asp[3]-Ala[4]-Ile[5]-Phe(pCl)[6]-Thr[7]-Asn[8]-Ser[9]-Tyr[10]-Arg[11]-Lys[12]-Val[13]-Leu[14]-Abu[15]-Gln[16]-Leu[17]-Ser[18]-Ala[19]-Arg[20]-Lys[21]-Leu[22]-Leu[23]-Gln[24]-Asp[25]-Ile[26]-Nle[27]-Ser[28]-Agm[29]
or [Nac[0],D-Arg[2],Phe(pCl)[6],Abu[15],Nle[27]]hGH-RH(1-28)Agm, is conducted in a stepwise manner using manual solid phase peptide synthesis equipment.

Boc-Agm-SPA-aminomethyl resin (California Peptide Co., Inc., California) (200 mg, 0.06 mmole) is deprotected with 50% TFA in CH_2Cl_2, neutralized with 5% DIEA in CH_2Cl_2, and washed as described in Table I. A solution of Boc-Ser(Bzl)-OH (55 mg, 0.18 mmole) in CH_2Cl_2 is shaken with the H-Agm-SPA-aminomethyl resin and DIC (31 μL, 0.2 mmole) in a manual solid phase peptide synthesis equipment for 1 hour. After wash and performance of the ninhydrin reaction to check for completeness of coupling, the cycle is repeated in a manner as described in Table I to build the peptide chain step-wise by adding the following protected amino acids in the indicated order on the resin:
Boc-Nle-OH, Boc-Ile-OH, Boc-Asp(OcHx)-OH, Boc-Gln-OH, Boc-Leu-OH, Boc-Leu-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Arg(Tos)OH, Boc-Ala-OH, Boc-Ser(Bzl)-OH, Boc-Leu-OH, Boc-Gln-OH, Boc-Abu-OH, Boc-Leu-OH, Boc-Val-OH, Boc-Lys(2-Cl-Z)-OH, Boc-Arg(Tos)OH, Boc-Tyr(2,6-diCl-Z)-OH, Boc-Ser(Bzl)-OH,

Boc-Asn-OH, Boc-Thr(Bzl)-OH, Boc-Phe(pCl)-OH, Boc-Ile-OH, Boc-Ala-OH, Boc-Asp(OcHx)-OH, Boc-D-Arg(Tos)OH, and Boc-Tyr(2,6-diCl-Z)-OH.

The protected amino acids (0.18 mmole each) are coupled with DIC (31 μL, 0.2 mmole) with the exceptions of Boc-Asn-OH and Boc-Gln-OH which are coupled with their preformed HOBt esters. After removal of the Boc protecting group from the alpha amino group of Tyr^1, the peptide is acylated by the symmetrical anhydride method. In this method, the symmetrical anhydride of 1-naphthylacetic acid is formed by reacting 123 mg (or 0.66 mmole) of 1-naphthylacetic acid with 60 μl (0.37 mmole) DIC; the resulting symmetrical anhydride is reacted with the peptide.

In order to cleave the peptide from the resin and deprotect it, the dried peptide resin (210 mg) is stirred with 0.5 mL m-cresol and 5 mL hydrogen fluoride (HF) at 0°C for 1 hour. After evaporation of the HF under vacuum, the remnant is washed with dry diethyl ether and ethyl acetate. The cleaved and deprotected peptide is dissolved in 50% acetic acid and separated from the resin by filtration. After dilution with water and lyophilization, 54 mg crude product is obtained.

60 mg of the GH-RH antagonist peptide is dissolved in AcOH/H_2O) and purified by RP-HPLC using the same procedure and equipments described in Example V. The product is judged to be substantially (>95%) pure by analytical HPLC. R_t = 13.52 min and k' = 0.819 (isocratic elution with 52% B). Confirmation of the structure is provided by amino acid analysis.

Peptides 19, 20, 21 and 38 are synthesized in the same manner as Peptide 18 except that they are acylated with the appropriate anhydride of isobutyric acid, bromopropionic acid, iodoacetic acid or formic acid respectively in place of Nac, to yield:

[Ibu^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nl ^{27}]hGH-RH(1-28)Agm P ptide 19

[BrProp^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm Peptide 20

[IAc^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm Peptide 21

[For^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm Peptide 38.

Peptides 23, 24, 25 and 26 are synthesized in the same manner as Peptide 18 except that Boc-Ser(Bzl)-OH^{28} is replaced with Boc-Asp(OcHx)-OH^{28} and they are acylated with the symmetrical anhydride of 1-naphtyl-acetic acid, 2-napthylacetic acid, 1-naphthoic acid, and anthraquinone-2-carboxylic acid, respectively, to yield:

[Nac^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27},Asp^{28}]hGH-RH(1-28)Agm Peptide 23

[2-Nac^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27},Asp^{28}]hGH-RH(1-28)Agm Peptide 24

[1-Npt^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27},Asp^{28}]hGH-RH(1-28)Agm Peptide 25

[Aqc^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27},Asp^{28}]hGH-RH(1-28)Agm Peptide 26.

Peptide 22 is synthesized in the same manner as Peptide 23 except that Boc-Tyr(2,6-diCl-Z)-OH^1 is replaced with Boc-His(Bom)-OH^1, to yield:

[Nac^0-His^1,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27},Asp^{28}]hGH-RH(1-28)Agm. Peptide 22

Peptide 27 is synthesized in the same manner as Peptide 18 except that Boc-Abu-OH^{15} is replaced with Boc-Ala-OH^{15}, to yield:

[Nac^0,D-Arg^2,Phe(pCl)^6,Ala^{15},Nle^{27}]hGH-RH(1-28)Agm Peptide 27.

Peptide 28 is synthesized in the same manner as Peptide 23 except that Boc-Asp(OcHx)-)H^3 is replaced with Boc-Gly-OH^3 to yield:

[Nac^0,D-Arg^2,Gly^3,Phe(pCl)^6,Abu^{15},Nle^{27},Asp^{28}]hGH-RH(1-28)Agm Peptide 28.

Peptides 29, 30, 31 and 33 are synthesized in the same manner as Peptide 23 exc pt that Boc-Phe-OH^6 is replaced with B c-Pro-OH^6, Boc-Pro-OH^6, Boc-hPhe-OH^6, and Boc-Ala-OH respectively and acylation is performed using the symmetrical anhydride f iodoacetic acid, isobutyric acid, iodoacetic acid and 1-naphthylacetic acid resp ctively, to yield:

[IAc^0,D-Arg^2,Pro^6,Abu^{15},Nle^{27},Asp^{28}]hGH-RH(1-28)Agm Peptide 29

[Ibu^0,D-Arg^2,Pro^6,Abu^{15},Nle^{27},Asp^{28}]hGH-RH(1-28)Agm Peptide 30

[IAc^0,D-Arg^2,hPhe^6,Abu^{15},Nle^{27},Asp^{28}]hGH-RH(1-28)Agm Peptide 31

[Nac^0,D-Arg^2,Ala^6,Abu^{15},Nle^{27},Asp^{28}]hGH-RH(1-28)Agm Peptide 33.

Peptide 32 is synthesized in the same manner as Peptide 18 except that Boc-Phe(pCl)-OH^6 is replaced with Boc-Nal-OH^6 to yield:
[Nac^0,D-Arg^2,Nal^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm Peptide 32.

Peptide 34 is synthesized in the same manner as Peptide 18 except that Boc-D-Arg(Tos)-OH^2 is replaced with Boc-D-Cit-OH^2, to yield:
[Nac^0,D-Cit^2,Phe(pCl)^6, Abu^{15},Nle^{27}]hGH-RH(1-28)Agm.

Peptide 35 is synthesized in the same manner as Peptide 34 except that acylation with the anhydride of 1-naphthylacetic acid is omitted to yield:
[D-Cit^2,Phe(pCl)^6, Abu^{15},Nle^{27}]hGH-RH(1-28)Agm.

Peptide 36 is synthesized in the same manner as Peptide 34 except that Boc-Phe(pCl)-OH^6 is replaced with Boc-Nal-OH respectively, to yield:
[Nac^0,D-Cit^2,Nal^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm.

Peptide 37 is synthesized in the same manner as Peptide 18 except that after removal of the Boc protecting group from the alpha amino group of Tyr^1, the peptide is *not* acylated.

EXAMPLE VIII

Biological Activity

The peptides of the present invention were tested in an in vitro and in vivo assay for their ability to inhibit the hGH-RH(1-29)NH_2 induced GH release.

Superfused Rat Pituitary System. The analogues were tested in vitro in a test described earlier (S. Vigh and A.V. Schally, Peptides 5:241-347, 1984) with modification (Z. Rekasi and A.V. Schally, P.N.A.S. 90:2146-2149, 1993).

Briefly, the cells are preincubated with peptides for 9 minutes (3mL) at various concentrations. Immediately after the incubation, 1 nM hGH-RH(1-29)NH_2 is administered for 3 minutes (1mL) [0 minute response]. To check the duration of the antagonistic effect of the analogue, 1 nM hGH-RH(1-29)NH_2 is applied 30, 60, 90, and 120 minutes later for 3 minutes [30, 60, 90, 120 min responses]. Net integral values of the GH responses are evaluated. GH responses are compared to and expressed as percent of the original GH response induced by 1 nM GH-RH(1-29)NH_2. The effect of the new antagonists are compared to that of [Ac-Tyr^1,D-Arg^2]hGH-RH(1-29)NH_2, the "Standard antagonist".

Growth Hormone Radio-immunoassay. Rat GH levels in aliquots of undiluted and diluted superfusion samples were measured by double-antibody radioimmunoassay using materials supplied by the National Hormone and Pituitary Program, Baltimore, Maryland. The results of RIA were analyzed with a computer program developed in our institute (V. Csernus and A.V. Schally, Harwood Academic (Greenstein, B.C. ed., London, pp. 71-109, 1991), hereby incorporated by reference. Inter-assay variation was less than 15% and intra-assay variation was less than 10%.

GH-RH Binding Assay. A sensitive radioreceptor binding assay was developed to determine the binding characteristics of the antagonists of GH-RH (G. Halmos, A.V. Schally et al., Receptor 3, 87-97, 1993), hereby incorporated by reference. The assay is based on binding of labelled [His^1,Nle^{27}]hGH-RH(1-32)NH_2 to rat anterior pituitary membrane homogenates. Iodinated derivatives of [His^1,Nle^{27}]hGH-RH(1-32)NH_2 are

prepared by the chloramine-T method (F.C. Greenwood et al., Biochemistry 89:114-123, 1963), hereby incorporated by reference. Pituitaries from male Sprague-Dawley rats (250-300 g) are used to prepare crude membranes. For saturation binding analyses, membrane homogenates are incubated with at least 6 concentrations of [His^1,^{125}I-Tyr^{10},Nle^{27}]hGH-RH(1-32) NH_2, ranging from 0.005 to 0.35 nM in the presence or absence of excess unlabelled peptide (1 μM). The pellet is counted for radioactivity in a γ-counter. The affinities of the antagonist peptides tested to rat pituitary GH-RH receptors are determined in competitive binding experiments. The final binding affinities are estimated by K_i (dissociation constant of the inhibitor-receptor complex) and are determined by the Ligand PC computer program of Munson and Rodbard as modified by McPherson. Relative affinities compared to [Ac-Tyr^1,D-Arg^2]hGH-RH(1-29)NH_2, the Standard antagonist, are calculated as the ratio of K_i of the tested GH-RH antagonist to the K_i of the Standard antagonist.

In Vivo Tests. Adult male Sprague-Dawley rats are anesthetized with pentobarbital (6mg/100g b.w., i.p.). Blood samples are taken from the jugular vein 30 min after the injection of pentobarbital. One group of 7 animals receives hGH-RH(1-29)NH_2 as control. Other groups of rats are injected with [Ac-Tyr^1,D-Arg^2]hGH-RH(1-29) NH_2 as Standard antagonist, or with one of the antagonist peptide 30 seconds prior to hGH-RH (1-29)NH_2, which is administered at dose of 2-3 μg/kg b.w. Blood samples are taken from the jugular vein 5 and 15 min after the injection of antagonists. GH levels are measured by RIA. Potencies of the antagonists are calculated by the factorial analysis of Bliss and Marks with 95% confidence limits and are based on the doses of 100 and 400 μg/kg b.w. of the Standard antagonist and 20 and 80 μg/kg b.w. of the antagonists tested. Statistical significance was assessed by Duncan's n w multipl range test.

Results in vitro. The results of the in vitro antagonistic activities tested in superfused rat pituitary system and binding assay are summarized in Table II and Table III, respectively. As can be seen from these data, acylation of the analogues with Nac or Ibu which contain D-Arg^2 or D-Cit^2 substitution combined with Phe(pCl)^6 or Nal^6, Abu^{15}, Nle^{27}, and Agm^{29} cause an immense increase in receptor binding as well as in inhibition of GH release in vitro. Antagonist peptides [Nac^0,D-Arg^2,pCl-Phe^6,Abu^{15}, Nle^{27}] hGH-RH(1-29)NH_2 (Peptide 1), [Nac^0-His^1,D-Arg^2,Phe(pCl)^6,Abu^{15}, Nle^{27}] hGH-RH(1-29)NH_2 (Peptide 5), Ibu^0,D-Arg^2,Phe (pCl)^6,Abu^{15},Nle^{27}] hGH-RH(1-28)Agm (Peptide 19) and [Nac^0, D-Arg^2, pCl-Phe^6,Abu^{15}, Nle^{27}] hGH-RH(1-28)Agm (Peptide 18) are the most effective antagonists in vitro. Peptides 1 and 18 are also extremely long acting in vitro: the inhibition of GH release is 90% (30 nM dose) of the control value 4.5 hours after the incubation in case of Peptide 1; and the inhibition of GH release by Peptide 18 is about 96% (30 nM dose) and 48% (3 nM dose) of the control value even 4.5 and 6 hours after the incubation, respectively. The receptor binding affinities of analogues Peptides 1, 5, and 19 are 82.56, 67.08, and 26.18 times greater respectively than that of the standard GH-RH antagonist.

Results in vivo. Table IV shows the serum GH levels in rats pretreated with GH-RH antagonists. Peptides 1 and 19 produce a significant greater and longer-lasting inhibition of the GH response to hGH-RH(1-29)NH_2 than the standard antagonist. In vivo experiments, Peptide 19 inhibits hGH-RH(1-29)NH_2-induced GH-release to greater extent and for a longer period of time than Peptide 1.

TABLE II

Inhibition of GH Release in Superfused Rat Pituitary System

Peptide	Dose (nM)	Inhibition of GH release (%) 0 min	30 min	60 min	120 min
Standard antagonist:	100	62.1	2.5	19	
1	100	23.3	93.9	89.3	
	30	96.1	95	92.1	88.8
	10	90.3	90	87.1	83.1
	3	18.1	31.5	17.1	
2	30	23.1	6		
3	30	80.7	16.4	0	
4	30	0	0	0	
5	30	92.6	86.4	81.4	64.5
6	30	17.9	0		
7	100	73.9	25	45	
	10	14.2	20.8	51.6	
8	30	59.1	0	7.3	14
9	100	90.7	79.5	76	
	30	88.4	46.5	43.9	32.1
10	300	2.5	21.5		
	100	29.4	49.7		
11	300	15.8	22.3		
12	100	87.9	51.8	42.4	
	30	81	35.6	0	
	10	65.5	33.6	8.5	
13	100	87.9	63.6	51.3	
	30	64.1	17.5	21	
	10	25.3	1.3	4.9	

Peptide	Dose (nM)	Inhibition of GH release (%) 0 min	30 min	60 min	120 min
14	100	38.9			
15	100	83.6	60.2	60.3	
	30	57.2	8.4	1.9	
	10	4.5	12.8	0	
16	100	7.8	18.7	14.7	
17	30	43.3	39.3	35.9	
18	30	83.6	93.9	89.3	98.9
	10	96.6	97.2	97.1	90.0
	3	77.6	83.4	75.3	58.8
	1	56.3	56.7	41.3	45.8
	0.3	11.0	45.0	15.6	13.5
19	100	95	74.7	36.7	
	30	82.7	40.7	9.6	
	10	70	18.2	13.9	
	3	62	16.4		
20	100	86.4	75	62.8	
	30	58	19.3	35.3	
	10	56.2	35.2	51.8	
21	300	89.3	32.9		
22	30	98.9	8.2	53.2	
	3	45.3	12.4	25.1	
23	30	89.3	85.1	71.6	63.8
	3	51.5	56.6	32.5	
24	30	83.6	64.4	6.7	60
	3	0	33.3	0	
25	30	84.5	32	42.7	32.8

Peptide	Dose (nM)	Inhibition of GH release (%) 0 min	30 min	60 min	120 min
26	30	64.9	48.9	42.3	
	3	24	31.2	21.6	
28	30	41.8	38.7	44	41.3
	3	0	22.1	5.1	
29	100	0	0		
30	300	36.2			
32	30	87.3	88.3	75.9	71.8
	3	35.9	37.1	43.4	
33	30	28.5	20.1	3.8	
34	30	91.2	87.4	84.8	
	3	70.4	50.5	40.6	
35	30	59.3	39.5	22.3	
37	30	97.5	67.3	58.4	62.1
	3	78.5	38.8		
38	30	94.5	0		
	3	49.2	0		

TABLE III

K_i values and relative affinities (R.A) of hGH-RH antagonists

Peptide	K_i (nM)	R.A.
Standard	3.22 ± 0.12	1
1	0.04 ± 0.01	82.56
5	0.05 ± 0.01	67.08
7	1.35 ± 0.02	2.39
8	0.91 ± 0.01	3.54
9	0.87 ± 0.1	3.72
12	0.30 ± 0.15	10.73
13	0.78 ± 0.06	4.13
15	0.73 ± 0.05	4.44
19	0.12 ± 0.04	26.18
20	0.99 ± 0.12	3.27

TABLE IV

Serum Growth Hormone Levels in Rats Pretreated with Different GH-RH Antagonists 5 Minutes Prior to Stimulation with GH-RH(1-29)NH_2

Treatment (intravenously)	Dose (μg/kg)	GH Levels (ng/mL)	POTENCY (measured against the Standard Antagonist)
Saline		89.0 ± 17.7	
GH-RH(1-29)NH_2	3.0	956.7 ± 113.6	
Standard antagonist	100.0	738.3 ± 34.7	
	400.0	439.7 ± 47.3*	
Peptide 19	20.0	451.8 ± 42.2*	
	80.0	155.0 ± 38.2*	
			18.90 95% Limits - 11.0-32.47
Peptide 1	20.0	641.2 ± 81.4	
	80.0	470.0 ± 46.1*	
			6.09 95% Limits - 3.11-11.96

* p,0.01 vs GH-RH(1-29)NH_2; Potenci s of the antagonists w re calculated by the factorial analysis of Bliss and Marks.

EXAMPLE IX

The experiment of Example VIII is repeated to evaluate the efficacy and duration of effect of GH-RH antagonist Peptide 18 in suppressing GH-RH(1-29)-stimulated serum growth hormone release in rats. Male Sprague-Dawley rats weighing 300-350 g were anesthetized with sodium pentobarbital (50mg/kg b.w.) and half of the initial pentobarbital dosage was given at 45 min intervals to maintain anesthesia. Twenty minutes after injection of pentobarbital, GH-RH antagonist Peptide 18 was administered intravenously in a dose of 80 μg/kg b.w. to the rats (0 time). Nine rats were used in each group. In order to stimulate GH release, bolus iv injections of GH-RH(1-29)NH_2 at a dose of 3μg/kg b.w. were given at 0 time and at 30 min after administration of the GH-RH antagonists. Blood samples were taken from the jugular vein 5 min after GH-RH(1-29)NH_2 injections. Serum GH levels were measured by radio-immunoassay. Statistical significance was assessed by Duncan's new multiple range test. The results of this experiment are shown in Table V.

TABLE V

Serum Growth Hormone Levels in Rats

Pretreated with GH-RH Antagonist Peptide 18

5 minutes prior to stimulation with GH-RH(1-29)NH_2 at a dose of 3μg/kg

Pretreatment (intravenously)	Dose (μg/kg)	GH Levels (ng/ml)
Saline		10.6 $\pm$ 0.02
GH-RH(1-29)NH_2	3.0	1650.6 $\pm$ 182.7
Peptide 18	80.0	1231.3 $\pm$ 81.3*

* $p < 0.05$ vs GH-RH(1-29)NH_2

GH-RH antagonist Peptide 18 injected at a dose f 80μg/kg inhibited GH-RH(1-29)NH_2-induced GH secretion by about 24% 5 minutes after its administration.

EXAMPLE X

Investigation of the Effect of Peptide 19 on the Growth of Human Osteosarcoma Cell Lines SK-ES-1 and MMNG/HOS Transplanted Athymic Nude Mice or Cultured In Vitro.

Methods: Male athymic nude mice bearing subcutaneously implanted Peptide 19 and MNNG/HOS tumors were treated for 4 and 3 weeks, respectively, with Peptide 19 administered from osmotic minipumps at a dose of 40μg/animal/day. Tumor volume and weight, mitotic index, apoptosis and Bromodeoxyuridine (BUdR) labeling index, an indicator of tumor cell proliferation were determined. The effect of Peptide 19 on IGF-I levels in serum, tumor and liver tissue were measured. Concentration of receptors of IGF-I was determined in tumor membrane fractions of both osteosarcomas. In addition, direct effects of Peptide 19 on DNA synthesis and proliferation of SK-ES-1 a nd MNNG/HOS cells, as well as on the secretion of IGF-I by these cell lines were evaluated in cell cultures.

Results: Growth of both osteosarcomas in nude mice was significantly inhibited by Peptide 19 (Figs.1 and 2). Growth inhibition of SK-ES-1 and MNNG/HOS tumors were reflected by a reduction in tumor volume of 64% and 49%, and a reduction in tumor weight of 62% and 47% respectively, after treatment with Peptide 19. Therapy with Peptide 19 also decreased tumors by 76% as measured by specific RIA. Receptor analyses demonstrated high affinity binding sites for IGF-I on membranes of both tumors. The concentration of IGF-I in liver tissue of nude mice not bearing tumors injected daily for 5 days with Peptide 19 was decreased by about 40% as compared to that in untr ated animals. In cell cultures, the proliferati n of SK-ES-1 and MNNG/HOS c lls, as well as the [^{3}H]thymidine incorporation into the DNA of both cell lin s w re strongly inhibited by

antagonist Peptide 19. The GH-RH antagonist also markedly reduced the autocrine secretion of IGF-I by both cell lines in vitro.

EXAMPLE XI

Study of the Effects of Peptide 19 on MXT Estrogen Independent Mouse Mammary Tumors

Female BDF mice were transplanted with 1mm 3 pieces of an MXT (3.2) estrogen independent breast cancer. Treatment with Peptide 19 started one day after transplantation as follows:

0.8μg/day s.c. once daily	5 mice
3.2μg/day s.c. once daily	5 mice

Tumor volume was measured on day 10, 14, and 18. The results are shown on the graph (Fig. 3). Antagonist Peptide 19 at either dose significantly inhibited the growth of breast cancer in mice.

EXAMPLE XII

Long Acting intramuscular injectable formulation (Sesame Oil Gel)

[Nac^0,D-Arg^2,$Phe(pCl)^6$,Abu^{15},Nle^{27}]hGH-RH(1-29)NH_2 (Peptide 1)	10.0 mg
Aluminum monostearate, USP	20.0 mg
Sesame oil g.s.	ad 1.0 ml

The aluminum monostearate is combined with the sesame oil and heated to 125°C with stirring until a clear yellow solution forms. This mixture is then autoclaved for sterility and allowed to cool. The hGH-RH antagonist Peptide 1 is then added aseptically with trituration. Particularly preferred antagonists are salts of low solubility, e.g., pamoate salts and the like. These exhibit long duration of activity.

EXAMPLE XIII

Aqueous Solution for Intramuscular Injection

[Nac^0,His^1-D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-29)NH$_2$ (Peptide 5)	500 mg
Gelatin, nonantigenic	5 mg
Water for injection g.s.	ad 100 ml

The gelatin and GHRH antagonist Peptide 19 are dissolved in water for injection, then the solution is sterile filtered.

EXAMPLE XIV

Long Acting IM Injectable-Biodegradable Polymer Microcapsules

Microcapsules are made from the following:

25/75 glycolide/lactide copolymer (0.5 intrinsic viscosity)	99%
[Ibu^0,D-Arg^2,Phe(pCl)^6,Abu^{15},Nle^{27}]hGH-RH(1-28)Agm^{29} (Peptide 19)	1%

25 mg of the above microcapsules are suspended in 1.0 ml of the following vehicle:

Dextrose	5.0%
CMC, sodium	0.5%
Benzyl alcohol	0.9%
Tween 80	0.1%
Water, purified q.s.	100.0%

The test results described herein are generally considered by those skilled in the art to be predictive of results in humans.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: The Administrators of the Tulane Educational Fund

(ii) TITLE OF INVENTION: ANALOGUES OF hGH-RH(1-29)NH2 HAVING ANTAGONISTIC ACTIVITY

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
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(D) STATE: NEW JERSEY
(E) COUNTRY: USA
(F) ZIP: 08837

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 29
(D) OTHER INFORMATION: /note= "Res 29 = Arg-NH_2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Tyr Ala Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Val Gln Leu
1 5 10 15

Gly Gln Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Met Ser Xaa
20 25

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1
(D) OTHER INFORMATION: /note= "Res 1 = Tyr or His"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 2
(D) OTHER INFORMATION: /note= "Res 2 = substituted D-Arg residues"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 27
(D) OTHER INFORMATION: /note= "Res 27 = Nle"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 29
(D) OTHER INFORMATION: /note= "Res 29 = Arg-NH_2"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Xaa Xaa Asp Ala Ile Phe Thr Asn Ser Tyr Arg Lys Val Leu Gly
1 5 10 15

Gln Leu Ser Ala Arg Lys Leu Leu Gln Asp Ile Xaa Ser Xaa
20 25

CLAIMS

1. A peptide having the formula:

X-R^1-R^2-R^3-R^4-R^5-R^6-Thr-R^8-Ser-Tyr-R^{11}-R^{12}-Val-Leu-R^{15}-Gln-Leu-Ser-R^{19}-R^{20}-R^{21}-Leu-Leu-Gln-Asp-Ile-R^{27}-R^{28}-R^{29} wherein

X is nil, H, Ac, IAc, BrProp, For, Ibu, Nac, 2-Nac, 1- or 2-Npt, 1- or 2-Npr or Aqc

R^1 is Tyr, His, Glt or Glu,

R^2 is D-Arg, D-Cit, D-Har, D-Lys or D-Orn,

R^3 is Asp, Ala or Gly,

R^4 is Ala or Gly,

R^5 is Ile, Ala or Gly,

R^6 is Phe, Ala, Pro, Tpi, Nal or Phe(Y), in which Y is F, Cl, Br, NO_2, CH_3 or OCH_3,

R^8 is Asn, Ser, Val, Ile, Ala, Abu, Nle, or Aib,

R^{11} is Arg, D-Arg or Cit,

R^{12} is Lys, D-Lys, Cit or Ala,

R^{15} is Gly, Ala, Abu or Gln,

R^{19} is Ala or Abu,

R^{20} is Arg, D-Arg or Cit,

R^{21} is Lys, D-Lys or Cit,

R^{27} is Met, Nle or Abu,

R^{28} is Ser, Asn, Asp or Abu,

R^{29} is Agm, Arg-NH_2, Arg-OH, Cit-NH_2, Cit-OH, Har-NH_2 or Har-OH,

provided that when R^1 is Glt, X is nil and when X is H, R^{15} is other than Gly,

and pharmaceutically acceptable acid addition salts thereof.

2. A peptide according to Claim 1 wh rein

X is H, Ac, IAc, BrProp, For, Ibu, Nac, 2-Nac, 1- or 2-Npt, 1- or 2-Npr, or Aqc,

R^1 is Tyr, His, or Glu,

R^2 is D-Arg or D-Cit,

R^3 is Asp or Gly,

R^6 is Phe, Phe(pCl), Tpi, Pro, hPhe, Nal or Ala,

R^8 is Asn or Aib,

R^{12} is Lys or Ala,

R^{15} is Abu or Ala,

R^{19} is Ala or Abu,

R^{27} is Nle,

R^{28} is Ser or Asp,

R^{29} is Agm or Arg-NH_2, and pharmaceutically acceptable acid addition salts thereof.

3. A peptide according to Claim 2 wherein

X is H, Ibu, For, Nac, 2-Nac, or 1-Npt,

R^1 is Tyr, His or Glu,

R^2 is D-Arg,

R^3 is Asp,

R^6 is Phe(pCl), Tpi, or Nal,

R^8 is Asn,

R^{12} is Lys,

R^{15} is Abu,

R^{19} is Ala, and

R^{28} is Ser.

4. A peptide according to Claim 3 wherein R^6 is Phe(pCl).

5. A peptide according to Claim 4 wherein X is Ibu or Nac, and R^1 is Tyr or His.

6. A peptide according to Claim 5 wherein X is Nac and R^1 is Tyr.

7. A peptid according to Claim 5 wherein X is Ibu.

8. A peptide according to Claim 5 wherein R^1 is His.

9. A peptide according to Claim 2 wherein X is Nac, R^2 is D-Cit and R^{29} is Agm.

10. A peptide according to Claim 3 wherein X is Nac, R^6 is Nal and R^{29} is Agm.

11. A peptide according to Claim 3, wherein X is For.

12. A peptide according to Claim 4 selected from the group consisting of peptides of the formula:

Nac^0-Tyr-D-Arg^2-Asp-Ala-Ile-Phe(pCl)^6-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu^{15}-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle^{27}-Ser-Arg-NH_2,

Nac^0-His^1-D-Arg^2-Asp-Ala-Ile-Phe(pCl)^6-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu^{15}-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle^{27}-Ser-Arg-NH_2,

Ibu^0-Tyr-D-Arg^2-Asp-Ala-Ile-Phe(pCl)^6-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu^{15}-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle^{27}-Ser-Agm, and

For^0-Ibu^0-Tyr-D-Arg^2-Asp-Ala-Ile-Phe(pCl)^6-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu^{15}-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle^{27}-Ser-Agm.

13. A peptide according to Claim 12 having the formula

Nac^0-Tyr-D-Arg^2-Asp-Ala-Ile-Phe(pCl)^6-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu^{15}-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle^{27}-Ser-Arg-NH_2.

14. A peptide according to Claim 12 having the formula

Nac^0-His^1-D-Arg^2-Asp-Ala-Ile-Phe(pCl)^6-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu^{15}-Gln-L u-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle^{27}-S r-Arg-NH_2.

15. A peptide according t Claim 12 having th formula Ibu^0-Tyr-D-Arg^2-Asp-Ala-Ile-Phe(pCl)^6-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu^{15}-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle^{27}-Ser-Agm.

16. A peptide according to Claim 12 having the formula For^0-Ibu^0-Tyr-D-Arg^2-Asp-Ala-Ile-Phe(pCl)^6-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu^{15}-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle^{27}-Ser-Agm.

17. A peptide according to Claim 3 selected from the group consisting of peptides of the formula Nac^0-Tyr-D-Arg^2-Asp-Ala-Ile-Phe(pCl)^6-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu^{15}-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle^{27}-Ser-Agm, Nac^0-Tyr-D-Arg^2-Asp-Ala-Ile-Nal^6-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu^{15}-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle^{27}-Ser-Agm, and Nac^0-Tyr-D-Cit^2-Asp-Ala-Ile-Phe(pCl)^6-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu^{15}-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle^{27}-Ser-Agm.

18. A peptide according to Claim 17 having the formula Nac^0-Tyr-D-Arg^2-Asp-Ala-Ile-Phe(pCl)^6-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu^{15}-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle^{27}-Ser-Agm.

19. A peptide according to Claim 17 having the formula Nac^0-Tyr-D-Arg^2-Asp-Ala-Ile-Nal^6-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu^{15}-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle^{27}-Ser-Agm.

20. A peptide according to Claim 17 having the formula Nac^0-Tyr-D-Cit^2-Asp-Ala-Ile-Phe(pCl)^6-Thr-Asn-Ser-Tyr-Arg-Lys-Val-Leu-Abu^{15}-Gln-Leu-Ser-Ala-Arg-Lys-Leu-Leu-Gln-Asp-Ile-Nle^{27}-Ser-Agm.

21. The use of a peptide according to Claim 1 for treating diabetic retinopathy.

22. The use of a peptide according to Claim 1 for treating diabetic nephropathy.

23. The use of a peptide according to Claim 1 for treating acromegaly.

24. The use of a peptide according to Claim 1 for treating the growth of MXT estrogen independent mouse mammary cancer.

25. The use of a peptide according to Claim 1 for treating human osteosarcomas.

26. A composition comprising a pharmaceutically effective amount of a peptide according to Claim 1 in a pharmaceutically acceptable carrier.

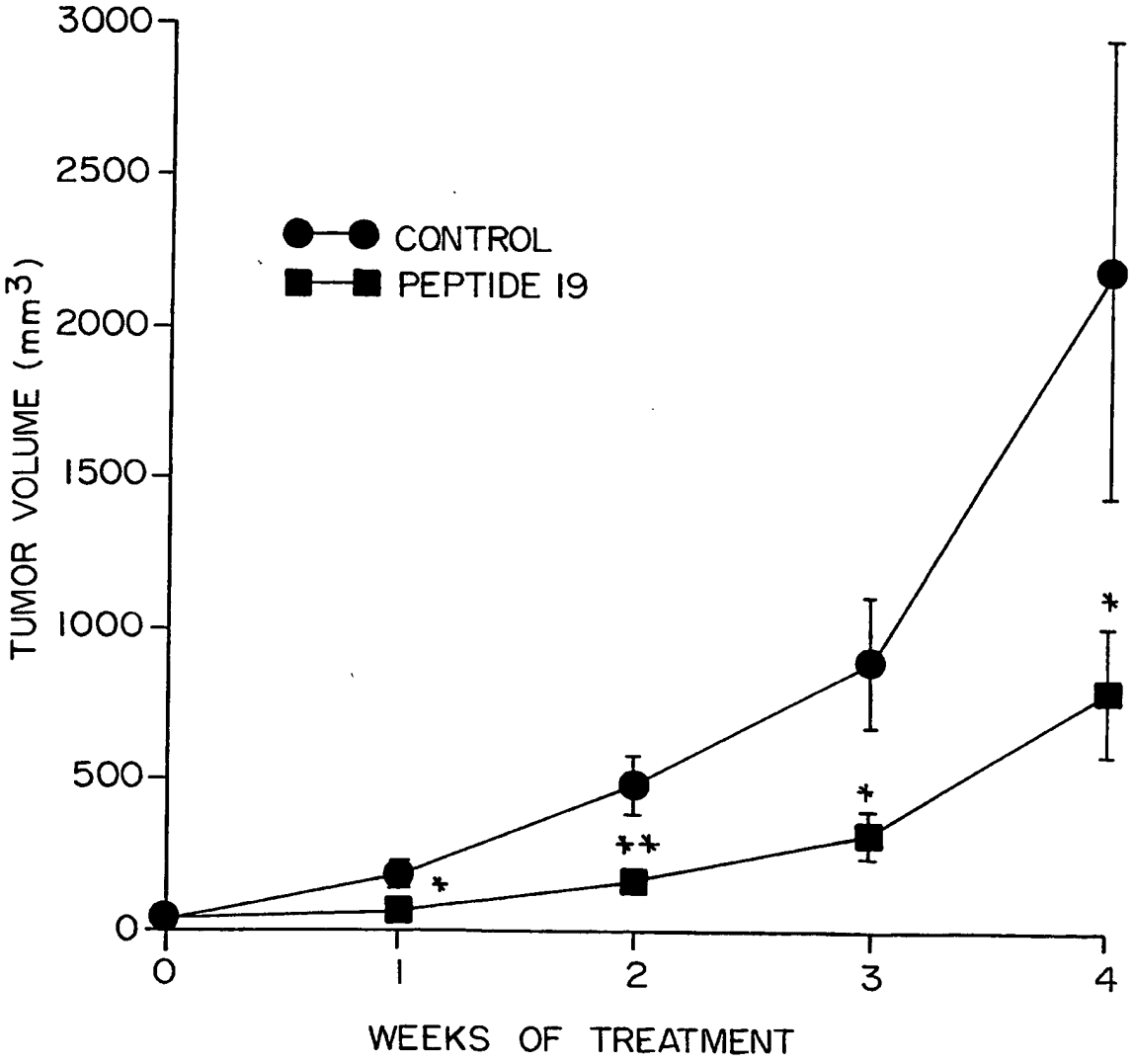
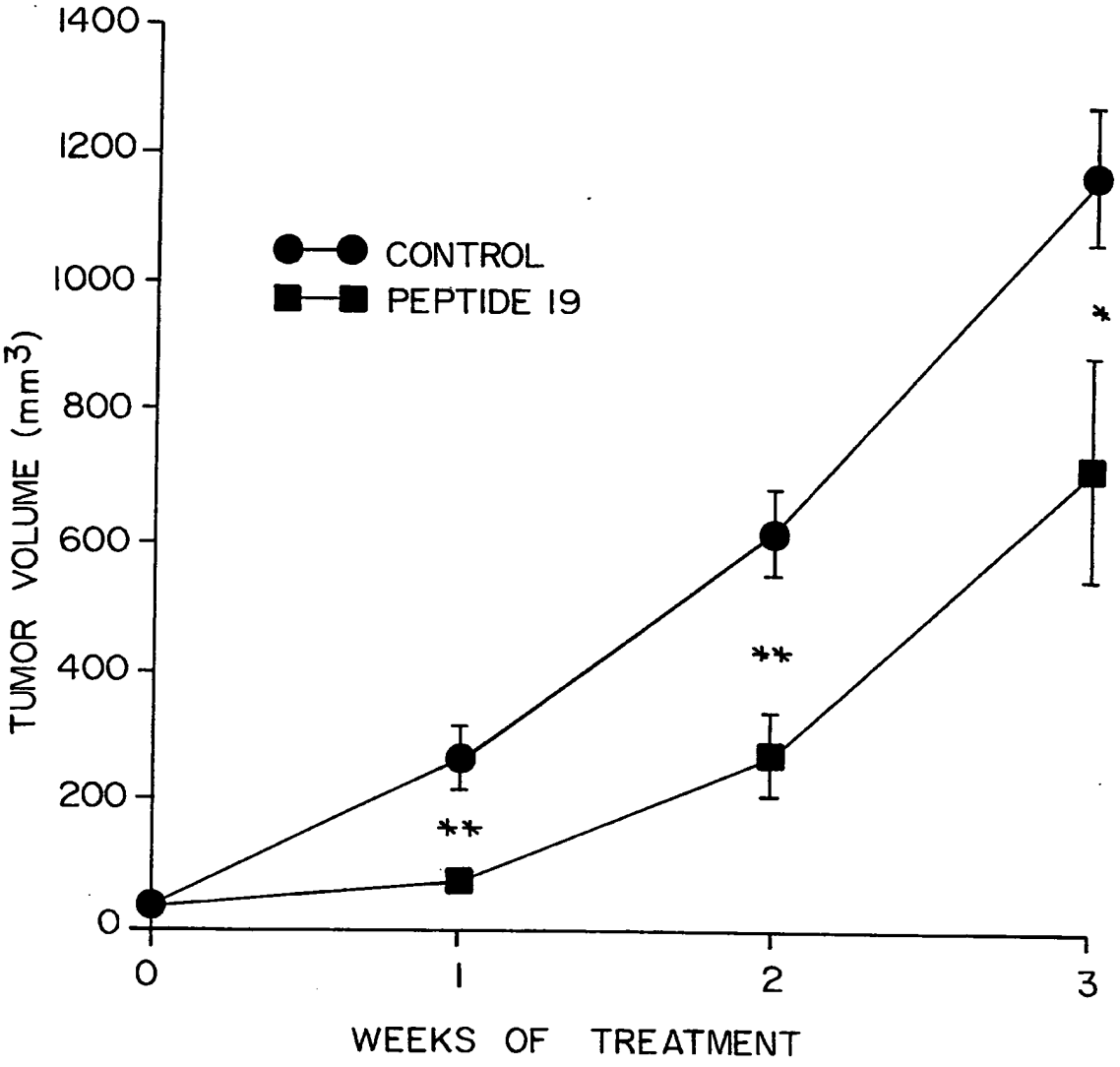

3000
2500
2000
1500
1000
500
0
TUMOR VOLUME (mm3)
CONTROL
PEPTIDE 19
*
**
*
*
0
1
2
3
4
WEEKS OF TREATMENT

FIG. 1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1200
1000
800
600
400
200
0
TUMOR VOLUME (mm3)
CONTROL
PEPTIDE 19
**
**
*
0
1
2
3
WEEKS OF TREATMENT

FIG. 2

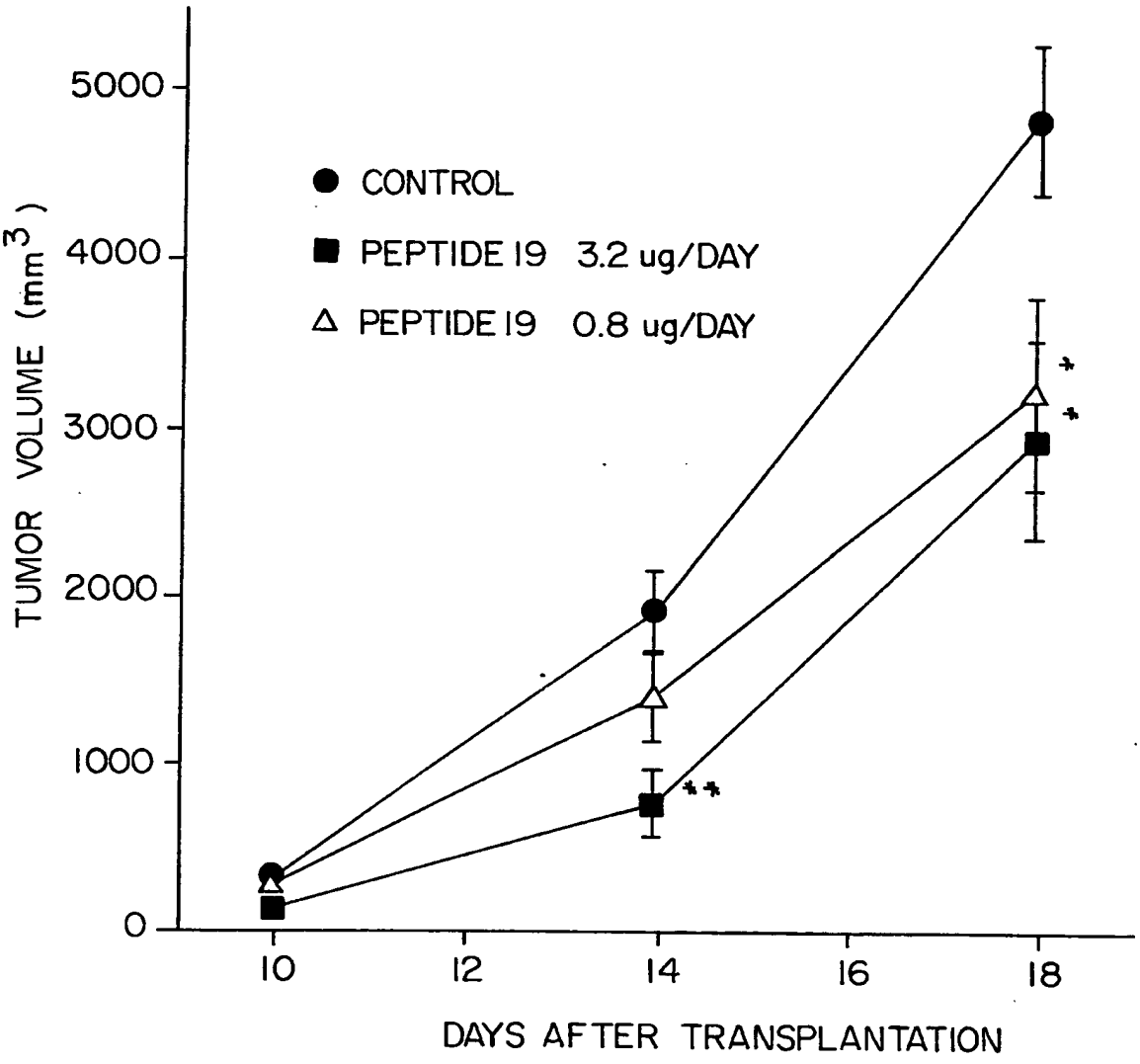
5000
4000
3000
2000
1000
0
TUMOR VOLUME (mm3)
CONTROL
PEPTIDE 19 3.2 ug/DAY
PEPTIDE 19 0.8 ug/DAY
10
12
14
16
18
DAYS AFTER TRANSPLANTATION

FIG. 3

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 94/13714

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/60 A61K38/25

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,91 16923 (THE ADMINISTRATORS OF THE TULANE UNIVERSITY EDUCATIONAL FUND) 14 November 1991 cited in the application see the whole document ---	1,2, 21-26
X	G.R.MARSHALL 'PEPTIDES,CHEMISTRY AND BIOLOGY; Proc.10th Am.Pept.Symp.,May 23-28,1987,St.Louis' 1988 , ESCOM , LEIDEN N.Ling et al: "Growth hormone-releasing factor analogs with potent antagonist activity" see page 484 - page 486 --- -/--	1,2, 21-26

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
11 April 1995	08.05.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Groenendijk, M

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BIOCHEM.BIOPHYS.RES.COMM, vol. 167,no. 1, 28 February 1990 pages 360-366, K.SATO ET AL 'Synthetic analogs of growth hormone-releasing-factor with antagonistic activity in vitro' see the whole document ---	1,21-26
A	EP,A,0 413 839 (THE ADMINISTRATORS OF THE TULANE EDUCATIONAL FUND) 27 February 1991 see page 2, line 39 - line 48 -----	1-12, 14-26

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/13714

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 21-25 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 94/13714

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9116923	14-11-91	AU-B-	651976	11-08-94
		AU-A-	7882291	27-11-91
		EP-A-	0527914	24-02-93
		JP-T-	6502618	24-03-94
EP-A-413839	27-02-91	NONE		